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Vaccin recombinant du birnavirus

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5 **Description**

[0001] The present invention is concerned with a bimavirus mutant, a vaccine comprising this mutant, a method for determining birnavirus infection in an animal, as well as with a test kit for carrying out this method.

5 [0002] Infectious bursal disease virus (IBDV) and Infectious pancreatic necrosis virus (IPNV) are members of the Birnaviridae family. Viruses in this family have a very similar genomic organisation and a similar replication cycle. The genomes of these viruses consist of 2 segments (A and B) of double-stranded (ds) RNA. The larger segment A encodes a polyprotein which is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4 (Hudson, P.J. et al., Nucleic Acids Res., 14, 5001-50012, 1986; Dobos P., Annual review of fish diseases 5, 25-54, 1995). VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of birnaviruses, and contains the antigenic regions responsible for the induction of neutralising antibodies. The VP4 protein appears to be a virus-coded protease that is involved in the processing of a precursor polyprotein of the VP2, VP3 and VP4 proteins. The larger segment A possesses also a second open reading frame (ORF), preceding and partially overlapping the polyprotein gene. This second open reading frame encodes a protein VP5 of unknown function that is present in IBDV infected cells (Mundt, E. et al., J. Gen. Virol., 76, 437-443, 1995).

10 [0003] The smaller segment B encodes VP1, a 90 kDa multifunctional protein with polymerase and capping enzyme activities (Spies, U. et al., Virus Res., 8, 127-140, 1987 and Spies, U. et al., J. Gen. Virol., 71, 977-981, 1990; Duncan R. et al., Virology 181, 541-552, 1991).

15 [0004] For IBDV, two serotypes exist, serotype 1 and 2. The 2 serotypes may be differentiated by virus neutralisation (VN) tests. Furthermore, subtypes of serotype 1 have been isolated. These so-called "variant" viruses of serotype 1 can be identified by cross-neutralisation tests (Diseases of Poultry, 9th edition, 1991, Wolfe Publishing Ltd, ISBN 0 7234 1706 7, Chapter 28, P.D. Lukert and Y.M. Saif, 648-663), a panel of monoclonal antibodies (Snyder, D.B. et al., Arch. Virol., 127, 89-101, 1992.) or RT-PCR (Jackwood, D.J., Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauschholzhausen, Germany, 155-161, 1994). Some of these subtypes of serotype 1 of IBDV have been described in literature for example: Classical, Variant-E, GLS, RS593 and DS326 strains (Van Loon, et al. Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauschholzhausen, Germany, 179-187, 1994).

20 [0005] Infectious Bursal disease (IBD), also called Gumboro disease, is an acute, highly-contagious viral infection in chickens that has lymphoid tissue as its primary target with a selective tropism for cells of the bursa of Fabricius. The morbidity rate in susceptible flocks is high, with rapid weight loss and moderate mortality rates. Chicks that recover from the disease may have immune deficiencies because of the destruction of the bursa of Fabricius which is essential to the defence mechanism of the chicken. The IBD-virus causes severe immunosuppression in chickens younger than 3 weeks of age and induces bursal lesions in chicks up to 3 months old.

25 [0006] For many years the disease could be prevented by inducing high levels of antibodies in breeder flocks by the application of an inactivated vaccine, to chickens that had been primed with attenuated live IBDV vaccine. This has kept economic losses caused by IBD to a minimum. Maternal antibodies in chickens derived from vaccinated breeders prevents early infection with IBDV and diminishes problems associated with immunosuppression. In addition, attenuated live vaccines have also been used successfully in commercial chicken flocks after maternal antibodies had declined.

30 [0007] Recently, very virulent strains of IBDV have caused outbreaks of disease with high mortality in Europe. The current vaccination programs failed to protect chicks sufficiently. Vaccination failures were mainly due to the inability of live vaccines to infect the birds before challenge with virulent field virus.

35 [0008] Eradication of the disease by other preventative measures than vaccination has not been feasible, because the virus is widely spread and because with currently administered live attenuated or inactivated IBDV vaccines it is not possible to determine whether a specific animal is infected with an IBDV field virus or whether the animal was vaccinated with an IBDV vaccine. In order to be able to start an eradication control programme for IBDV it is highly desirable that the possibility exists to discriminate between animals vaccinated with an IBDV vaccine and those infected with a field virus so as to be able to take appropriate measures, i.e. remove infected flocks, to reduce spreading of the virulent field virus. The introduction of, for example, a serologically identifiable marker can be achieved by introducing a mutation in genes encoding non-essential (glyco)proteins of the IBDV which still give rise to the production of antibodies in an infected host animal. A marker vaccine for Aujeszky's disease and companion diagnostic tests have proven their practical value in the control of this disease. Whereas such control programs for other viral infectious diseases in animals are under development, until the present invention a vaccine based on an IBDV vaccine strain which would fit in IBDV control programs has not been described yet.

40 [0009] Mundt and Kölner (Annual Meeting of the German Federal Research Institute for virus diseases in animals, March 1997) disclose the construction of an IBDV mutant that fails to express a VP5 protein as a result of a genetically engineered mutation in the start codon of the VP5 gene. This mutation comprises the substitution of one nucleotide in the start codon of the VP5 gene.

[0010] The present invention provides a birnavirus mutant as defined in the claims which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome.

[0011] Preferably, the birnavirus mutant is an IBDV mutant or an IPNV mutant, the IBDV mutant being most preferred, in particular an IBDV mutant derived from a serotype 1 IBD virus is provided by the present invention.

5 [0012] It is demonstrated that an IBDV mutant that is not able to produce a VP5 protein is still able to infect poultry and to replicate in the infected host animals *in vivo*, i.e. evidence is provided that the gene encoding the VP5 protein is a non-essential gene. Example 3 and 4 show that VP5⁻ IBDV can be re-isolated from organs of animals infected with the IBDV mutant and that the IBDV mutant induces a protective immune response in the infected animals.

10 [0013] Moreover, it has been established herein that part of the normal anti-IBDV immune response in poultry is directed to the VP5 region. This is rather surprising as the VP5 protein is considered to represent a non-structural viral protein (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) and the immune response in an animal against a viral pathogen is usually elicited against the structural (glyco)proteins of the virus. These findings make the IBDV mutant and other birnavirus mutants according to the present invention a suitable vaccine candidate for a marker vaccine. Such a marker vaccine provides the possibility to determine whether animals are infected with a wild-type birnavirus, e.g. IBDV, or with a vaccine virus.

15 [0014] Additionally, it has been found that the VP5 protein is involved in the expression of virulence of the birnaviruses, in particular of IBDV, and that the inability of the virus mutants to produce the native VP5 protein leads to an attenuation of the virus.

20 [0015] With the term "which is not able to produce a native VP5 protein" is meant that the birnavirus mutant produces a polypeptide that can be distinguished by serological tests from the native VP5 protein, or does not produce a VP5 protein at all. For example, in the former case, the birnavirus mutant produces only a fragment of the native birnavirus VP5 protein which lacks one or more immunogenic epitopes.

25 [0016] Preferably, the birnavirus mutant according to the invention produces no VP5 protein upon infection of a host cell.

30 [0017] As described above, the genomic organisation of the birnaviruses is well established: the IBDV and IPNV genome comprises a large segment A and a smaller segment B. The segment A of IBDV comprises a large open reading frame (ORF) encoding a polyprotein of about 110 kDa (VP2-VP4-VP3). The gene encoding the VP5 protein is identified in the prior art, and defined herein, as the small ORF on segment A of the birnavirus genome which precedes and partially overlaps the polyprotein encoding ORF (Bayliss et al., J. Gen. Virol. 71, 1303-1312, 1990; Spies et al., J. Gen. Virol. 71, 977-981, 1990; Havarstein L.S. et al., J. Gen. Virology 71, 299-308; 1990; Dobos et al., 1995, *supra*; Figures 1-3 herein and SEQ ID No.'s 1-7). The mutation introduced in the VP5 gene is such that it does not prevent the expression of the polyprotein.

35 [0018] SEQ ID No. 1 comprises the full length cDNA nucleotide sequence of segment B of IBDV strain P2, as well as the amino acid sequence of the VP1 protein encoded by segment B (see also SEQ ID. No. 2). SEQ ID No. 3 and 5 depict the full length cDNA sequence of segment A of IBDV strain D78 and the coding region of the VP5 protein and the polyprotein, respectively. SEQ ID 3 and 4 also show the amino acid sequence of the D78 VP5 protein. SEQ ID No. 5 and 6 show the amino acid sequence of the polyprotein VP2-VP4-VP3 of D78. SEQ ID No. 7 shows the 5'-end of segment A of strain D78, including the mutations introduced in the VP5 coding region. SEQ ID No. 8 shows the nucleotide sequence of segment B of strain D78 and the amino acid sequence of the D78 VP1 protein. The genomic organisation of both segments is also shown in Figure 1.

40 [0019] The ORF coding for VP5 is conserved in all hitherto published segment A sequences. The IBDV ORF encodes 145 amino acids resulting in a calculated molecular mass of 16.5 kDa. The nucleotide sequence of the ORF encoding the VP5 protein of IBDV strain D78 used herein is shown in SEQ ID No. 3 and 4. Natural variations may exist between individual IBDV isolates. These natural variations result from small differences in the genomes of these viruses. The nucleotide sequence of the segment A, including the nucleotide sequence of the VP5 gene for many IBDV isolates have been described in the prior art (Vakharia et al., Avian Diseases 36, 736-742, 1992; Bayliss et al., J. Gen. Virol. 71, 1303-1314, 1990; Hudson et al., Nuc. Acid Res. 14, 5001-5012, 1986; Schnitzler et al., J. Gen. Virol. 47, 1563-1571, 1993; Kibenge et al., J. Gen. Virol. 71, 569-577, 1990 and Virology 184, 437-440, 1991; Mundt et al., Virology 209, 10-18, 1995; Lana et al., Virus Genes 6, 247-259, 1992; Vakharia et al., Virus Res. 31, 265-273, 1994; Brown et al., Virus Res. 40, 1-15, 1996). The amino acid sequence of the VP5 protein from serotype I IBDV strains display a homology of at least 95% with the VP5 amino acid sequence shown in SEQ ID No. 3 and 4, whereas the homology between serotype II VP5 sequence and the amino acid sequence shown in SEQ ID No. 3 and 4 is at least 75%. Therefore, a preferred IBDV mutant according to the present invention is an IBDV mutant wherein the mutation is introduced in the VP5 gene having a homology of at least 75%, in particular at least 95% on the amino acid sequence level with the VP5 amino acid sequence shown herein.

45 [0020] Preferably an IBDV mutant according to the present invention is derived from any of the classical or variant (e.g. variant E or GLS) IBDV vaccine strains, such as those currently used in the field. Such suitable IBDV strains include the IBDV vaccine strains present in the commercially available vaccines: D78, PBG 98, LZ 228E, 89-03 (Intervet

International B.V.), Bursine 2 (Fort Dodge Animal Health) and S 706 (Rhone Mérieux).

[0021] A particular preferred IBDV mutant according to the invention is derived from the D78 strain comprising a VP5 gene encoding a protein having the amino acid sequence shown in SEQ ID No. 3 and 4.

[0022] Alternatively, the parent birnavirus strain for the virus mutant according to the invention is a virulent birnavirus field strain. It is found herein that the VP5 protein is a factor associated with virulence, and that the absence of the native VP5 protein in a birnavirus results in an attenuated form of the virus.

[0023] Preferably the invention provides a birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the part of the VP5 gene which does not overlap with the large ORF encoding the polyprotein.

[0024] In particular, the birnavirus mutant according to the invention comprises a mutation in the 5'-end of the VP5 gene spanning nucleotides 1-30, preferably 1-20, more preferably 1-10. Most preferred is an birnavirus mutant having a mutation in nucleotides 1-3 of the VP5 gene.

[0025] A mutation is understood to be a change of the genetic information in the VP5 gene with respect to the genetic information present in this region of the genome of naturally occurring birnavirus producing native VP5 protein. The mutation is, for example, a nucleic acid substitution, deletion, insertion or inversion, or a combination thereof.

[0026] In a preferred embodiment of the present invention a birnavirus mutant is provided wherein the mutation is a substitution of one or more nucleotides. In particular, a nucleic acid substitution is introduced in the start codon, as a result of which the new codon encodes an amino acid different from methionine or represents a stop codon, preferably the nucleic acid substitution comprises at least two of the nucleotides of the start codon.

[0027] A further birnavirus mutant according to the invention comprises a substitution of one or more nucleotides in a codon(s) different from the start codon resulting in one or more stop codons, preferably in the 5'-end of the VP5 gene as defined above, if desired in addition to a substitution in the start codon as described above. Preferably, the birnavirus mutant comprises a stop codon in this region of the VP5 gene in each of the three reading frames.

[0028] Such a preferred birnavirus mutant may be an IBDV mutant having a mutation in the start codon, the fourth and the sixth codon of the VP5 gene, preferably resulting in the mutated codons shown in SEQ ID No. 7 and Figure 3.

[0029] Alternatively, a birnavirus mutant is provided wherein the mutation is a deletion. In particular, the deletion comprises less than 20, less than 10 or less than 5 nucleotides. Preferably, the deletion comprises a total number of nucleotides not dividable by three, resulting in a shift of the reading frame.

[0030] Preferably the deletion comprises one or more nucleotides of the start codon of the VP5 gene.

[0031] In an alternative embodiment of the present invention a bimavirus mutant is provided wherein the mutation comprises the insertion of a heterologous nucleic acid sequence in the birnavirus genome. A heterologous nucleic acid sequence is a nucleic acid sequence normally not present at the specific insertion site of the particular virus species.

[0032] The heterologous nucleic sequence to be incorporated into the birnavirus genome is a nucleic acid fragment which either encodes a polypeptide or is a non-coding sequence. The nucleic acid fragment can be derived from any source, e.g. viral, eukaryotic, prokaryotic or synthetic, including oligonucleotides suitable for the interruption of the expression of the VP5 gene.

[0033] A suitable oligonucleotide for the interruption of the VP5 expression may comprise three translational stop codons in each of the possible reading frames in both directions, in addition to one or more appropriate restriction enzyme cleavage sites useful for the insertion of a second heterologous nucleic acid sequence. The length and nucleotide sequence of such a non-coding heterologous nucleic acid sequence is not critical, but preferably varies between 8-50 nucleotides.

[0034] In a further embodiment of the present invention a bimavirus mutant is provided which can be used not only for the preparation of a vaccine against infection by a specific birnavirus, but also against other poultry or fish infectious diseases. For example, a vector vaccine based on such an IBDV mutant offers the possibility to immunise against other avian pathogens by the expression of antigens of these avian pathogens within infected cells of the immunised host. Such an IBDV vector according to the present invention can be obtained by inserting a heterologous nucleic acid sequence encoding a polypeptide heterologous to the IBDV in the VP5 gene as defined herein.

[0035] The heterologous nucleic acid sequence may encode an antigen of an avian pathogen such as Newcastle disease virus, Infectious bronchitis virus, Marek's disease virus, avian encephalomyelitis virus, avian reovirus, avian influenza virus, chicken anaemia virus, *Salmonella spp.*, *E.coli*, and *Eimeria spp.*

[0036] Furthermore, an IBDV mutant according to the invention comprises in addition to the mutation in the VP5 gene, a mutation in the VP2 gene, wherein this gene expresses a chimeric protein comprising neutralising epitopes of more than one antigenic type of IBDV (e.g. classic, Variant-E and/or GLS). Preferably, such a mutant comprises the relevant protective VP2 epitopes of a variant GLS strain and classic strain. In particular, the mutated VP2 gene is a GLS VP2 gene comprising a nucleic acid sequence fragment encoding the B69 epitope. The construction of such a mutated VP2 genes is described in Snyder et al., *Avian Diseases* 38, 701-707, 1994. Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immuno-modulators such as lymphokines, interferons or cytokines, may be incorporated into the VP5 gene. The heterologous nucleic acid sequence may also encode a screenable marker, such as *E. coli* β -galactosidase or *E. coli* β -glucuronidase.

5 [0037] The construction of birnavirus mutants, in particular of IBDV mutants according to the present invention can be achieved by means of the recently established infectious cRNA system for IBDV (Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996). This reverse genetics system opens the possibility to introduce mutations in the RNA genome of the IBD virus, in particular in the VP5 gene. The most important step in this reverse genetics system is to provide full length cDNA clones of the segments A and B of IBD virus. cDNA constructs comprising the segment A or B, including the nucleotides of the 5'- and 3'- ends of both these segments can be generated according to the method described by Mundt and Vakharia (1996, *supra*). Additionally, these constructs comprise a RNA polymerase promoter operably linked to either of the segments. The promoter can be the promoter for the T7, SP6 or T3 polymerase, the T7 promoter being preferred. Mutations can be introduced into the VP5 gene by means of methods generally known in the art for this purpose. In particular, the mutation(s) are introduced by means of site directed mutagenesis.

10 [0038] For example, in a first step a cDNA fragment is provided comprising at least a substantial part of the VP5 gene. In the next step suitable primer pairs are designed and hybridised with the VP5 sequence containing fragment. The 5'-primer comprises in addition to sequences complementary to the VP5 sequence, nucleotides which harbour 15 the desired mutation, e.g. a mutation which changes the ATG start codon to an AGG (arginine) codon. Moreover, the 5'-primer is provided with an upstream nucleotide sequence representing a suitable restriction enzyme cleavage site which allows the restoring of the complete 5'-end non-coding sequence. Subsequently, the new mutated fragment is 20 amplified using PCR and the new fragment is introduced in the starting sequence by replacing the native nucleic acid sequence using appropriate restriction enzymes. In the next step plus-sense transcripts of the segment A and B are generated *in vitro* with (T7) RNA polymerase, after which the synthetic transcripts are purified using conventional RNA purification techniques. The recombinant IBDV mutant according to the invention is obtained after transfection of suitable cells (e.g. VERO cells, QM-7 cells or CEC cells) with the synthetic RNA transcripts of both segments of the IBDV genome, if desired in the presence of transfection-enhancing compositions, such as Lipofectin. Finally the recombinant IBDV is harvested from the supernatant of the transformed cells.

25 [0039] Methods for introducing a mutation in the birnavirus genome are described herein, but are also generally used in the art (Mundt and Vakharia, 1996, *supra*; Current Protocols in Molecular Biology, eds.: F. M. Ausubel et al., Wiley N.Y., 1995 edition, pages 8.5.1.-8.5.9.)

30 [0040] Further to the unexpected finding by the present inventors that the VP5 ORF of IBDV is a non-essential region of the IBDV genome, it has also been found that an IBDV mutant according to the present invention is able to induce a protective immune response, i.e. animals immunised with a vaccine comprising the IBDV mutant are protected against 35 virulent challenge. Moreover, it has been found that anti-sera of animals infected with naturally occurring IBDV comprise antibodies directed to the non-structural VP5 protein and that these antisera can be distinguished from anti-sera derived from animals infected with an IBDV mutant according to the present invention. In addition, it has been found that the IBDV mutant as described above is attenuated if compared with the parent IBD virus which is able to produce the native VP5 protein.

40 [0041] Therefore, another aspect of this invention is a vaccine for use in the protection of animals against birnavirus infection comprising the birnavirus mutant as characterised above, together with a pharmaceutical acceptable carrier or diluent. In particular, the vaccine according to the invention is a vaccine for use in the protection of poultry against infectious bursal disease comprising the IBDV mutant described above.

45 [0042] The birnavirus mutant according to the present invention can be incorporated into the vaccine as live or inactivated virus.

[0043] A vaccine according to the invention can be prepared by conventional methods such as for example commonly used for the commercially available live- and inactivated IBDV vaccines. Briefly, a susceptible substrate is inoculated with an IBDV mutant according to the invention and propagated until the virus replicated to a desired infectious titre 50 after which IBDV containing material is harvested.

[0044] Every substrate which is able to support the replication of IBD viruses can be used in the present invention, including primary (avian) cell cultures, such as chicken embryo fibroblast cells (CEF) or chicken kidney cells (CK), mammalian cell lines such as the VERO cell line or the BGM-70 cell line, or avian cell lines such as QT-35, QM-7 or LMH. Usually, after inoculation of the cells, the virus is propagated for 3-10 days, after which the cell culture supernatant is harvested, and if desired filtered or centrifuged in order to remove cell debris.

55 [0045] Alternatively, the IBDV mutant is propagated in embryonated chicken eggs. In particular, the substrate on which these IBD viruses are propagated are SPF embryonated eggs. Embryonated eggs can be inoculated with, for example 0.2 ml IBDV mutant containing suspension or homogenate comprising at least 10² TCID₅₀ per egg, and subsequently incubated at 37 °C. After about 2-5 days the IBD virus product can be harvested by collecting the embryo's and/or the membranes and/or the allantoic fluid followed by appropriate homogenising of this material. The homogenate can be centrifuged thereafter for 10 min at 2500 x g followed by filtering the supernatant through a filter (100 µm).

[0046] The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilised form and additionally contains a pharmaceutically acceptable carrier or diluent cus-

5 tomary used for such compositions. Carriers include stabilisers, preservatives and buffers. Suitable stabilisers are, for example SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

[0047] If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are the same as mentioned below.

10 [0048] Although administration by injection, e.g. intramuscular, subcutaneous of the live vaccine according to the present invention is possible, the vaccine is preferably administered by the inexpensive mass application techniques commonly used for IBDV vaccination. For IBDV vaccination these techniques include drinking water and spray vaccination.

[0049] Alternative methods for the administration of the live vaccine include in ovo, eye drop and beak dipping administration.

15 [0050] In another aspect of the present invention a vaccine is provided comprising the birnavirus mutant in an inactivated form. The major advantage of an inactivated vaccine is the extremely high levels of protective antibodies of long duration that can be achieved.

20 [0051] The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general, this can be achieved by chemical or physical means. Chemical inactivation can be effected by treating the viruses with, for example, enzymes, formaldehyde, β -propiolactone, ethylene-imine or a derivative thereof.

25 If necessary, the inactivating compound is neutralised afterwards. Material inactivated with formaldehyde can, for example, be neutralised with thiosulphate. Physical inactivation can preferably be carried out by subjecting the viruses to energy-rich radiation, such as UV light or γ -rays. If desired, after treatment the pH can be adjusted to a value of about 7.

[0052] A vaccine containing the inactivated birnavirus mutant can, for example comprise one or more of the above-mentioned pharmaceutically acceptable carriers or diluents suited for this purpose.

30 [0053] Preferably, an inactivated vaccine according to the invention comprises one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

[0054] The vaccine according to the invention comprises an effective dosage of the birnavirus mutant as the active component, i.e. an amount of immunising birnavirus material that will induce immunity in the vaccinated birds against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

35 [0055] Typically, the live vaccine according to the invention can be administered in a dose of 10^2 - 10^9 TCID₅₀ infectious dose₅₀ (TCID₅₀) per animal, preferably in a dose ranging from $10^{5.0}$ - $10^{7.0}$ TCID₅₀, and an inactivated vaccines may contain the antigenic equivalent of 10^5 - 10^9 TCID₅₀ per animal.

[0056] Inactivated vaccines are usually administered parenterally, e.g. intramuscularly or subcutaneously.

40 [0057] Although, the IBDV vaccine according to the present invention may be used effectively in chickens, also other poultry such as turkeys, guinea fowl and partridges may be successfully vaccinated with the vaccine. Chickens include broilers, reproduction stock and laying stock.

[0058] The age of the animals receiving a live or inactivated vaccine according to the invention is the same as that of the animals receiving the conventional live- or inactivated IBDV vaccines. For example, broilers (free of maternally derived antibodies-MDA) may be vaccinated at one-day-old, whereas broilers with high levels of MDA are preferably vaccinated at 2-3 weeks of age. Laying stock or reproduction stock with low levels of MDA may be vaccinated at 1-10 days of age followed by booster vaccinations with inactivated vaccine on 6-8 and 16-20 weeks of age.

45 [0059] The invention also includes combination vaccines comprising, in addition to the IBDV or IPNV mutant according to the invention, one or more immunogens derived from other pathogens infectious to poultry or fish, respectively.

[0060] Preferably, the combination vaccine additionally comprises one or more vaccine strains of infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome (EDS) virus, turkey rhinotracheitis virus (TRTV) or reovirus.

50 [0061] In addition to a marker vaccine for birnaviruses, the availability of an appropriate diagnostic test is an essential requirement for the application of a birnavirus eradication control programme. Such a diagnostic test is provided herein and comprises a method for determining IBDV infection in poultry and IPNV infection in fish, i.e. it provides a method for distinguishing an animal in the field vaccinated with a vaccine as described above, from an animal infected with a naturally-occurring IBDV or IPNV.

[0062] Therefore, the present invention provides a method for the detection of birnavirus infection, in particular for the detection of IBDV infection in an animal comprising the step of examining a sample of the animal for the presence of VP5 antibodies or antigens. The animal is an animal from the field and is in particular an avian species, preferably

5 a chicken. The sample coming from the animal may be any sample in which IBDV antibodies or antigens are present, e.g. a blood, serum or tissue sample, the serum sample being preferred.

10 [0063] A preferred method for determining birnavirus infection in an animal is a method for the detection of antibodies against the VP5 protein, comprising the steps of:

15 (i) incubating a sample suspected of containing anti-birnavirus antibodies, with VP5 antigen,
 (ii) allowing the formation of antibody-antigen complex , and
 (ii) detecting the presence of the antibody-antigen complex.

20 [0064] The design of this immunoassay may vary. For example, the immunoassay may be based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labelled antibodies; the labels may be, for example, enzymes, fluorescent-, chemiluminescent-, radio-active- or dye molecules.

25 [0065] Suitable methods for the detection of the VP5 antibodies in the sample include the enzyme-linked immunosorbent assay (ELISA), immunofluorescent test (IFT) and Western blot analysis.

30 [0066] In an exemplifying ELISA, the wells of a polystyrene micro-titration plate are coated with VP5 antigen. Next, the wells of the coated plates are filled with chicken serum and serial dilutions are made. After incubation, chicken anti-VP5 protein serum antibodies are determined by detecting antibody (monoclonal or polyclonal) with the same specificity as the coated one, but which is labelled (e.g. with biotin). The labelled antibody will occupy the free antigens that have not been occupied by anti-VP5 antibodies in the chicken serum. For example, horse radish peroxidase coupled to avidin may be added and the amount of peroxidase is measured by an enzymatic reaction. If no antibodies against VP5 are present in the chicken serum sample then a maximum absorption is obtained. If the serum contains many antibodies against VP5 then a low absorption is expected. Alternatively, after the incubation with chicken serum, the amount of antibodies present in the serum that bound to the VP5 antigen may be determined directly by using an anti-chicken conjugate followed by the enzymatic reaction.

35 [0067] In a sandwich ELISA the wells of a polystyrene micro-titration plate can be coated with a monoclonal antibody directed against the VP5 protein. Next, the wells of these coated plates are incubated with VP5 antigen. After the antigen is captured, the wells are filled with the chicken serum and serial dilutions are made. Subsequently, the protocol as described above may be followed. This test can also be carried out by using polyclonal serum against VP5 instead of the coated monoclonal antibodies.

40 [0068] In another diagnostic test (Western blot analysis), the VP5 antigen (containing) material is subjected to SDS-PAGE. Next, the separated proteins are electroblotted onto nitro-cellulose membrane. Thereafter, the membranes can be cut into lanes and the lanes are incubated with the chicken serum. The presence of VP5 antibodies in the sample can be determined by examination whether antibodies bound to the VP5 antigen, for example by using an anti-chicken conjugate followed by an enzymatic reaction. If antibodies against VP5 are present then a band at about 17 kDa is identifiable.

45 [0069] The VP5 antigen may be any VP5 protein (fragment) comprising material which allows the formation of the VP5 antigen-VP5 antibody complex. Preferably, the VP5 antigen comprises the expression product of a conventional recombinant host cell or virus, e.g. such as E.coli expressed VP5 (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) or baculovirus expressed protein (Vakharia et al., Vaccine 12, 452-456, 1994; Vakharia et al., J. Gen Virol. 74, 1201-1206, 1993). In a further embodiment of the present invention a diagnostic test kit is provided which is suitable for performing the diagnostic test according to the invention as described above.

50 [0070] In particular, a diagnostic test kit is provided which comprises in addition to the components usually present, the VP5 antigen (if desired coated onto a solid phase) as the immunological reagent. Other components usually present in such a test kit include, biotin or horseradish peroxidase conjugated antibodies, enzyme substrate, washing buffer etc.

[0071] To determine birnavirus VP5 antigen in a test sample from an animal in the field, VP5-specific antibodies are used as the immunological reagent, preferably fixed to a solid phase. The test sample is added, and after an incubation time allowing formation of the antibody-antigen complex, a second labelled antibody may be added to detect the complex.

EXAMPLESExample 1.5 Construction and analysis of recombinant VP5⁻ IBD virus**Construction of full length VP5⁻ clone of IBDV segment A.**

[0072] To construct a VP5-negative IBDV, the *Eco*RI site immediately following the 3'-end of the full length cDNA of strain D78 segment A (pUC19FLAD78; Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996) was deleted. An *Eco*RI - *Kpn*I fragment containing the T7 polymerase binding site followed by the complete segment A sequence was excised and inserted into *Eco*RI - *Kpn*I cleaved vector pUC18 after inactivation of the unique *Nde*I within the vector sequence resulting in plasmid pAD78/EK. Thereafter, the genomic region encompassing the initiation codon for VP5 was amplified in two pieces using primers A1F5' and VP5MutR, and VP5MutF and A2R, respectively (see Table 1 for sequence and location of primers). PCR fragments were cloned separately and were subsequently fused via a unique *Afl*II site which had been created by mutations within respective primers (see Fig. 2). An *Eco*RI - *Nde*I fragment containing the T7 polymerase binding site, and the 5'-part of segment A including the introduced mutations was excised and used to substitute the wild-type *Eco*RI - *Nde*I fragment in pAD78/EK to yield plasmid pAD78/VP5⁻. Of the three mutations introduced one altered the initiation methionine codon for VP5 into an arginine codon (Fig. 2).

20

Table 1: Sequence of oligonucleotide primers used for generating mutant constructs.

a	Nucleotide sequence	Orientation	Designation	Nucleotide no.
	<u>AGAGAATTCTAATACGACTCACTATAGGA</u> <u>TACGATCGGTCTGAC</u>	+	A1F5'	1-18
	<u>TGGGCCTGTCACTGCTGTACATGT</u> <u>CATTGCTCTGCAGTGTGTAGTGAGC</u>	-	A2R	716 - 740
	<u>CTACAAACGCTATCCTTAAGGGTTAGTA</u> <u>GAG</u>	+	VP5MutF	338 - 362
	<u>CTCTACTAACCCTTAAGGATAGCGTTGT</u> <u>AG</u>	-	VP5MutR	80 - 109

40

a) Underlined nucleotides denote virus specific nucleotides. T7 promotor sequences are marked in italics. Mutated nucleotides are bold and orientation of the primer is shown for sense (+) and antisense (-). Primer positions are given according to the published sequence of serotype I strain P2 (Mundt et al., Virology 209, 209-218, 1995).

45

[0073] **Virus recovery from cRNA.** For *in vitro* transcription of RNA plasmids pAD78/EK, pAD78/VP5⁻ and pBP2 (Fig. 2) were linearized by cleavage with *Bsr*GI and *Pst*I, respectively. Treatment of linearized DNA, transcription and purification of RNA, and transfection were carried out as described by Mundt and Vakharia (1996, *supra*) with the exception that secondary CEC were used for the transfection experiments. Three days after transfection a CPE was visible in CEC. Cells were freeze/thawed, centrifuged at 700 x g to eliminate cellular debris, and the resulting supernatants were filtrated through 0.45 µm filters and stored at -20°C. For the transfection experiments full length cDNA clones of segment A of strain D78 capable of expressing (pAD78/EK) or unable to express VP5 (pAD78/VP5⁻) were transcribed into synthetic RNA and cotransfected with segment B full length cRNA into CEC. Resulting virus progeny IBDV/EK and IBDV/VP5⁻ was further characterised.

55

[0074] **Analysis of transfection progeny by immunofluorescence and Radioimmunoprecipitation assay**

(RIPA). VP5 was expressed in E.coli as described in Mundt et al. (J. Gen. Virol. 76, 437-443, 1995). Rabbit monospecific polyclonal anti serum and mouse monoclonal antibodies against VP5 were prepared according to standard protocols. Vero cells infected with IBDV/VP5-, IBDV/EK, and non-infected cells, respectively, were incubated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and with anti-VP5 mAb DIE 7, and stained with fluoresceine-conjugated secondary antibodies. Both antisera and the monoclonal antibody recognised IBDV antigens in the cytoplasm of IBDV/EK infected cells. In contrast, whereas the anti-IBDV serum readily detected viral antigens in IBDV/VP5- infected cells, neither the monospecific anti VP5-serum nor the monoclonal anti-VP5 antibody exhibited specific reactivity. None of these immunological reagents reacted with non-infected controls.

[0075] To analyse viral proteins expressed during replication lysates of radioactively labelled CEC infected with IBDV/VP5- (Fig 4, lanes 1-3) and IBDV/EK (Fig. 4, lanes 4-6) were immunoprecipitated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and mAb DIE 7. Non-infected CEC were used as control (Fig. 4, lanes 7-9). IBDV/EK (lane 4) as well as IBDV/VP5- (lane 1) infected CEC showed viral proteins VP2, VP3, and VP4 after precipitation with rabbit anti-IBDV serum. The rabbit anti-VP5 serum (lane 5) and mAb DIE 7 (lane 6) precipitated VP5 with a molecular mass of 21 kDa only from IBDV/EK infected cells. No specific reactivity was detectable in IBDV/VP5- infected CEC after precipitation with rabbit-anti VP5 (lane 2) as well as the VP5 specific mAb DIE 7 (lane 3). Non-infected CEC showed no specific reactivity (lanes 7-9).

[0076] **Replication of IBDV/VP5- in CEC.** To assay replication of IBDV/VP5- in more detail one step growth was analysed (Fig. 5). Confluent secondary CEC were infected with IBDV/EK and IBDV/VP5- with $10^{7.2}$ TCID₅₀, respectively. Immediately after overlaying the infected cells with 5 ml growth medium, supernatant from one infected CEC tissue plate of each virus was removed and stored at -20°C (0 h p.i.). Remaining tissue culture plates were further incubated and 4h, 8h, 16h, 24h, and 48h p.i. supernatants were removed and stored at -20°C. Supernatants were centrifuged and titrated according to standard methods. The TCID₅₀ at the different time points after infection showed that the VP5 expressing virus (IBDV/EK) replicated faster than the virus mutant lacking VP5 (IBDV/VP5-). 16 h after infection IBDV/EK showed a 100-fold higher than IBDV/VP5- (Fig. 5). However, at 48 h p.i. IBDV/VP5- reached a titre of $10^{7.2}$ TCID₅₀/ml which was similar to IBDV/EK ($10^{7.45}$ /ml).

[0077] **Preparation of recombinant IBDV VP5-2.** Plasmid pAD78/VP5-2 was prepared by techniques similar to those described above. The nucleotide sequence of part of the mutated VP5 gene is shown in SEQ ID No. 7 and Figure 3. A restriction enzyme fragment harbouring the mutations was used to substitute the wild-type EcoRI - NdeI fragment in pAD78/EK. An outline of the protocol for the preparation of the recombinant plasmid is shown in Figure 3. The organisation of pBD78 is also depicted in Figure 3. The recombinant virus was prepared as described above, except for the fact that segment B of strain D78 (SEQ ID No. 8) was used and QM-7 cells were used for the transfection experiment.

Example 2

Identification of VP5 protein in different IBDV strains

[0078] Different strains of IBDV were investigated for the expression of the VP5-gene. This was done by making use of the immuno-fluorescence technique (IFT). Chicken embryo fibroblasts grown in microtiterplates were infected with different IBDV strains. Three to 5 days after incubation at 37°C cells were fixed with 70% ethanol, then treated with polyclonal rabbit anti IBDV serum (R1928), polyclonal rabbit anti VP5 serum (R α VP5) or monoclonal antibody directed against VP5 (DIE7), respectively. Binding of the poly- or monoclonal antibodies to the different IBDV strains was visualised by making use of a fluorescence labelled conjugate (goat-anti-rabbit or goat-anti-mouse). The results are shown in Table 2:

Table 2

Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.					
IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	R α VP5	DIE7
I	Classical	D78	+	+	+
I	Classical	228TC	+	+	+
I	Classical	PBG98	+	+	+
I	Classical	Ram0404	+	+	+
I	Classical	IBDV/EK	+	+	+
I	Classical	IBDV/VP5-	+	-	-

Table 2 (continued)

Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.					
IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	R α VP5	DIE7
I	GLS	GLS	+	+	+
I	Variant-E	8903	+	+	+
II	TY89	TY89	+	+	+

[0079] From these data it can be concluded that the different strains of IBDV belonging to different sero- and subtypes do express the VP5-gene. Furthermore, the recombinant VP5⁺ IBDV vaccine strain can be differentiated from field and vaccine viruses, thereby enabling the recombinant VP5⁺ virus to be used as a marker vaccine.

Example 3

In vivo testing of the recombinant VP5⁺ and VP5⁻ IBDV vaccines in comparison with a commercial available live IBDV vaccine.

[0080] **Preparation of IBDV vaccine.** Primary chicken embryo fibroblast (CEF) cells were prepared at a final concentration of 2×10^6 /ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 25 ml of this cell suspension 0.1 ml IBDV/EK or IBDV/VP5⁻ virus (having an infectious titre of about $3.0 \log_{10}$ TCID₅₀/ml) was added. After incubation for 5 days in a high-humidity incubator at 37°C, the total suspension was used in the animal experiment without further purification. The infectious titre of the supernatant was $10^{7.1}$ TCID₅₀/ml.

[0081] **Animal experiment.** In this study the potency of different vaccines (VP5 positive strain IBDV/EK and a VP5 negative strain IBDV/VP5⁻, and the commercial available IBDV vaccine Nobilis strain D78, Intervet International B.V., NL) was investigated. SPF chicks of 3 weeks old were treated as indicated in the treatment schedule.

Treatment Schedule:

Days after vaccination	Groups			
	1	2	3	4
00	IBDV/EK	IBDV/VP5 ⁻	D78	-
03	x	x1	x	x
07	x,bl	x1,bl	x,b	x,bl
14	x,bl	x,bl	x,bl	x,bl
20	x,bl	x,bl	x,bl	x,bl
21	ch	ch	ch	ch
24	x	x	x	x
31	+	+	+	+

VP5⁺ Bursal disease vaccination with VP5 positive vaccine clone, eye-drop route, dose $10^{4.6}$ TCID₅₀/animal, 0.1 ml/animal.

VP5⁻ Bursal disease vaccination with VP5 negative vaccine clone, eye-drop route, dose $10^{5.9}$ TCID₅₀/animal, 0.1 ml/animal.

D78 Bursal disease vaccination with IBDV VACCINE NOBILIS STRAIN D78, eye-drop route, one field dose.

ch Challenge with Bursal disease virus, Farragher strain F52/70, eye-drop route, dose $10^{2.0}$ CID₅₀/animal, 0.1 ml/animal.

bl Serological examination; VN-test and/or Western blotting.

x Histological examination (H.E. staining) and MCA-8 ELISA on bursae.

x1 Histological examination (H.E. staining) and MCA-8 ELISA on bursae and reisolation of virus from bursa of Fabricius.

+ Clinical examination and after 10 days histological examination of the bursa.

5 **Detection of virus in the bursa of Fabricius.**

[0082] Three, 7, 14 and 20 days after eye-drop vaccination, animals were sacrificed and blood and bursae obtained. The presence of virus in the bursa was determined with an enzyme-linked immunosorbent assay (ELISA) making use of the monoclonal antibody 8 (MAB-8). MAB-8 is directed specifically against IBDV. Data are depicted in Table 3.

10 [0083] Furthermore, 3 and 7 days after vaccination, bursae from animals of group 2 were investigated for the presence of the recombinant VP5⁻ virus. For that purpose bursae were homogenised and cultured on chicken embryo fibroblasts. The presence of the VP5⁻ virus was determined by IFT using polyclonal rabbit sera against IBDV or VP5 or monoclonal antibodies against VP5. From 13 out of 15 bursae (87%) investigated, VP5⁻ virus could be reisolated and identified (positive for R1928 and negative for R α VP5 and DIE7). This indicates that the virus upon animal passage is still VP5⁻, indicating that the virus is stable and does not revert to VP5⁺. Furthermore, by using the different poly- and monoclonal antibodies VP5⁻ vaccine virus can be discriminated from all other vaccine and/or field IBDV viruses. Therefore, the VP5⁻ vaccine may be used as a marker vaccine.

15 [0084] Three days after challenge no virus could be detected in groups 1, 2 and 3 with the MCA-8 ELISA. In contrast, all animals of group 4 (non-vaccinated control group) contained challenge virus in the bursa of Fabricius, 3 days after challenge. The results show that animals vaccinated with recombinant VP5⁺ (group 1), recombinant VP5⁻ (group 2) and IBDV vaccine Nobilis D78 (group 3) were protected against severe challenge.

20 **Table 3**

Individual data for detection of virus in the bursa of Fabricius with the MCA-8 ELISA at different days after vaccination or challenge.						
	Days after vaccination→				Days after challenge	
	3	7	14	20		
Group↓	Virus detection by ELISA↓					Protection↓
1 VP5 ⁺	2/8*	1/7	0/2	0/3	0/5	100%
2 VP5 ⁻	0/8	0/7	0/2	0/3	0/5	100%
3 D78	1/8	6/7	0/2	0/3	0/5	100%
4 -	0/8	0/7	0/2	0/3	5/5	0%

25 *Number of positive bursae per total number tested.

30 **Detection of lesions in the bursa of Fabricius.**

[0085] The microscopic average lesion score induced by the different IBDV (recombinant) vaccines or the challenge virus are depicted in Table 4.

35 [0086] Before challenge, animals vaccinated with the recombinant VP5⁺ IBDV vaccine (group 1) or vaccinated with IBDV vaccine Nobilis D78 (group 3) showed mild to moderate lesions in the bursa. Three days after challenge only chronic lesions were observed in the bursa of Fabricius, indicating that the animals of groups 1 and 3 were protected against challenge. Furthermore, 10 days after challenge only very mild lesions (0-20% lymphocytic depletion) were observed in the bursa of the animals vaccinated with VP5⁺ recombinant IBDV vaccine or with Nobilis vaccine D78. In contrast animals not vaccinated and challenged showed severe lesions 10 days after challenge. In other words all animals (100%) of groups 1 and 3, vaccinated with the VP5⁺ recombinant IBDV vaccine or with Nobilis vaccine D78 were protected against severe challenge.

40 [0087] Three, 7, 14 and 20 days after vaccination and 3 and 10 days after challenge with the recombinant VP5⁻ IBDV vaccine, animals of group 2 showed no to hardly any lesions (0-20% lymphocytic depletion) in the bursa. All animals of group 2, vaccinated with the VP5⁻ recombinant IBDV vaccine, were protected against severe challenge. When animals vaccinated with the recombinant VP5⁻ IBDV vaccine are compared to animals of groups 1 or 3 (vaccinated with a recombinant VP5⁺ or commercial available vaccine) the recombinant VP5⁻ vaccine induces less lesions and therefore, is safer, milder than the vaccines tested in this experiment.

45 [0088] Three days post-challenge, all non-vaccinated animals of group 4 showed severe acute lesions in the bursa (total lymphocyte depletion, score 5.0). Ten days after challenge, all animals (17 out of 17 animals) showed total lymphocytic depletion, indicating that these animals were not protected against severe challenge. Animals that died after challenge, all showed severe lesions in the bursa of Fabricius. It was concluded that control group 4 was not protected against severe challenge indicating that the test conditions were optimal.

Table 4

Average bursal lesion score at different days after vaccination or challenge. The average lesion score is calculated as follows: all lesion scores from the animals per group on a certain day are added. This number is then divided by the total number of animals investigated in that group on that day. Individual scores range from 1 to 5. Score 0 = no lymphocytic depletion, score 1 = 0 - 20%; score 2 = 20 - 40%; score 3 = 40 - 60%; score 4 = 60 - 80% and score 5 = 80 - 100 % lymphocytic depletion (total lymphocytic depletion).

	Days after vaccination→				Days after challenge→		
	3	7	14	20	3	10	
Group↓	Bursal lesions score↓						Protection↓
1 VP5 ⁺	0.8	2.9	1.0	1.0	1.0 ^c	0.6	100%
2 VP5 ⁻	0.0	0.0	0.5	0.0	0.0 ^c	0.1	100%
3 D78	0.1	2.4	3.5	2.0	2.8 ^c	1.1	100%
4 -	0.0	0.0	0.0	0.0	5.0 ^a	5.0	0%

^a Acute lesions

^c Chronic lesions

Serological response.

[0089] The serological response of the animals was determined by measuring the ability of blood serum to neutralise a classical infectious bursal disease virus strain in a virus neutralising (VN) test. Serum was investigated 3, 7, 14 and 20 days after vaccination. The average neutralising titres are shown in Table 5.

[0090] The results show that recombinant IBDV vaccine VP5⁺ applied to chickens of group 1 induced a good and high serological response 20 days after vaccination which is comparable to the serological response of the chickens vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3). The recombinant IBDV vaccine VP5⁻ applied to chickens of group 2 induced also a good serological response. A titre of 9.4 log₂ was observed 20 days after vaccination. The serological response induced by the recombinant VP5⁻ IBDV vaccine was delayed when compared to the serological response induced by the recombinant IBDV VP5⁺ vaccine or the commercial IBDV vaccine Nobilis strain D78.

[0091] The non-vaccinated group 4 showed no serological response to IBDV.

Table 5

Average IBDV-VN-titres for groups 1 to 4 at different days after vaccination, expressed as log ₂ of the dilution.				
Group	Days after vaccination			
	3	7	14	20
1 VP5 ⁺	≤ 1.0 ± 0.0	7.1 ± 1.7	10.2 ± 1.4	11.9 ± 1.8
2 VP5 ⁻	≤ 1.0 ± 0.0	2.1 ± 1.7	6.3 ± 2.9	9.4 ± 1.4
3 D78	≤ 1.0 ± 0.0	5.2 ± 2.8	10.3 ± 1.3	11.6 ± 1.5
4-	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0

Serological differentiation between antisera.

[0092] The serological response against VP5 was investigated by making use of western blot analysis. For this purpose the VP5 protein was expressed in the *E. coli* or baculo expression system. The expressed proteins were separated by SDS PAGE. Next the proteins were electroblotted onto a nitro-cellulose membrane. Thereafter, the membrane was cut into lanes and the lanes were incubated with rabbit anti-VP5 serum, chicken serum directed against VP5⁺ recombinant vaccine, chicken serum directed against VP5⁻ recombinant vaccine or negative serum from SPF chickens. Data are summarised in Table 6. As can be seen from Table 6, the VP5⁻ serum does not induce a serological response against VP5. In contrast the rabbit anti-VP5 serum and chicken serum directed against VP5⁺ recombinant vaccine do recognise the VP5-protein and thus induces a serological response against VP5. This indicates that chicken serum may be used to investigate if animals are exposed to a virus that expresses the VP5 protein (e.g. field virus) or

to the VP5⁺ recombinant vaccine.

Table 6

5	Western blot analysis. Serum from animals vaccinated with VP5 ⁺ or VP5 ⁻ recombinant vaccine as well as SPF chicken serum and anti VP5-rabbit serum were investigated for their reaction with the VP5-protein.	
10	Identification of serum sample	Immuno-blot
15	VP5 ⁺ vaccinated animal, serum sample 20d after vaccination	positive
20	VP5 ⁻ vaccinated animal, serum sample 20d after vaccination	negative
25	Non-vaccinated control, serum sample at 20d	negative
30	Rabbit anti VP5 serum	positive

15 Mortality and clinical signs.

[0093] None of the animals vaccinated with VP5⁺ IBDV vaccine (group 1), vaccinated with recombinant VP5⁻ IBDV vaccine (group 2) or vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3), died or showed clinical signs of infectious bursal disease after challenge, indicating that the animals were protected against severe challenge. All animals in the non-vaccinated control group were not protected against severe challenge.

Example 4

In vivo testing of the recombinant VP5-2 vaccine

[0094] **Preparation of the IBDV vaccines.** Primary chicken embryo fibroblasts (CEF) cells were prepared at a final concentration of 2×10^6 /ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 15 ml of this cell suspension 0.1 ml IBDV/VP5-2 (D78/D78/VP5⁻) virus was added. After incubation for 6 days in a high humidity incubator at 37°C, the supernatant was titrated. The infectious titre of the supernatant was $10^{8.2}$ TCID₅₀/ml. For the second animal experiment the supernatant was diluted to result in a vaccine dose of $10^{5.5}$ TCID₅₀/animal and for the first animal experiment the supernatant was diluted to result in a vaccine dose of $10^{4.0}$ TCID₅₀/animal or $10^{5.0}$ TCID₅₀/egg.

[0095] **First animal experiment.** The effect of the vaccine is assessed by measurement of the serological response and resistance to challenge obtained from administering a challenge virus at the age of 14 days. The vaccine ($10^{5.0}$ TCID₅₀/egg or $10^{4.0}$ TCID₅₀/animal of D78/D78/VP5⁻) was applied *in ovo* or intramuscularly at day old. Microscopic lesions in the bursa were investigated, 3 and 10 days after challenge. Protection against challenge was determined and the serological response at the age of 14 days old was determined with the VN-test.

40 1. Average microscopic lesion score in the bursa 3 and 10 days after challenge.

Days post challenge	Group		
	<i>In ovo</i>	Day old	None-vaccinated
3	3.3	0.0	5.0
10	0.2	0.0	5.0

2. Protection after challenge

	Group		
	<i>In ovo</i>	Day old	None-vaccinated
% protection	91.6	100	0

3. Serological response against IBDV

	Group		
	<i>In ovo</i>	Day old	None-vaccinated
VN-titre	6.4 ± 1.7	6.4 ± 1.3	<4.0 ± 0.0

VN-titre is expressed as log2 of the dilution. Animals with a titre <4.0 log2 are considered negative

Conclusions

[0096]

- 1 The D78/D78/VP5⁻ strain is a highly attenuated IBD-virus
- 2 The virus strain is very mild
- 3 The virus can induce a serological response
- 4 The virus can induce protection
- 5 The virus strain can be applied by intramuscular injection to 1 day old SPF chickens and *in ovo* to 18-days-old embryonated SPF-eggs

[0097] **Second animal experiment.** The effect of the vaccine is assessed by measurement of the serological response against IBDV and resistance to challenge obtained from administering a challenge virus, 21 days after administering the Gumboro vaccine. The vaccine ($10^{5.5}$ TCID₅₀/animal of D78/D78/VP5⁻) was applied via the intramuscular route to 14 days old SPF-chickens. Three, 7, 14, and 20 days after vaccination and 3 days after challenge Bursa, spleen, thymus, liver, duodenum, pancreas, caecal tonsils and harderian gland were investigated for microscopic lesions. Ten days after challenge Bursae were investigated for microscopic lesions. Sera were tested in the VN-test. And mortality was scored after challenge.

1. Percentage mortality after challenge:	
	Mortality after challenge
Vaccinated group	0%
Control group	50%

2. Microscopic lesions of the vaccinated group before and after challenge:

(continued)

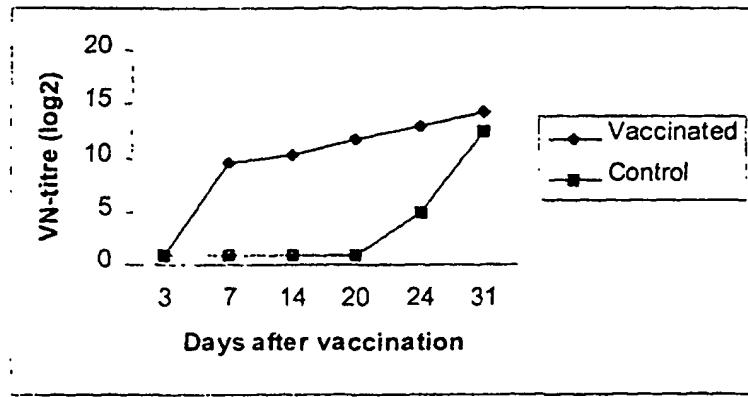
2. Microscopic lesions of the vaccinated group before and after challenge:								
Days post	Bursa	Spleen	Thymus	Liver	Duodenum	Pancreas	Ceacal	Harderian
Vaccinat.							Tonsils	Gland
14	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
24	0,A	0	0	0	0	0	0	0
31	0,A	ND	ND	ND	ND	ND	ND	ND

A = None vaccinated animals showed a lymphocytic depletion score of 5.0 (100%) and 4.25, 3 and 10 days after challenge, respectively. ND = not done.

15

20

3. Serological response after vaccination:



35

Conclusions

[0098]

40

1. The D78/D78/VP5⁻ strain is a highly attenuated IBD-virus
2. The virus strain is very mild and does not induce lesions in organs
3. The virus can induce a serological response
4. The virus can induce protection

45

LEGENDS TO THE FIGURES

[0099] Figure 1 Genomic organization of segment A and segment B of IBDV. The numbers indicate the nucleotide positions of the start, end and coding region on the segments.

[0100] Figure 2 Construction of genomic cDNA clones for the preparation of IBDV/VP5⁻. Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBP2 contains the complete strain P2 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5⁻ altering the methionine start codon for VP5 into arginine and creating an artificial *Afl* II cleavage site. Recombinant plasmids were linearized with the underlined restriction enzymes, followed by T7 polymerase transcription.

[0101] Figure 3 Construction of genomic cDNA clones for the preparation of IBDV/VP5⁻². Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBD78 contains the complete strain D78 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5⁻ altering the methionine start codon for VP5 into glutamic acid and creating an artificial *Bst* BI cleavage site. Further mutations were introduced in the arginine and glutamine codon. Recombinant plasmids were linearized with the underlined restriction

enzymes, followed by T7 polymerase transcription.

[0102] Figure 4 Radioimmunoprecipitation of proteins from CEC infected cells with recombinant IBDV. CEC infected cells with IBDV/VP5⁻ (lanes 1-3), IBDV/EK (lanes 4-6) and uninfected controls were immunoprecipitated with rabbit anti-IBDV serum (lanes 1, 4, 7), rabbit anti-VP5 serum (lanes 2, 5, 8) and mAb DIE 7 (lanes 3, 6, 9). Position of molecular mass markers (M) is indicated. Location of the viral proteins VP2, VP3, VP4 and VP5 are marked.

[0103] Figure 5 Replication kinetics of IBDV/EK and IBDV/VP5⁻. Infectious titers of supernatants (vertical axis) are determined at the times indicated.

SEQUENCE LISTING

[0104]

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Azko Nobel N.V.
- (B) STREET: Velperweg 76
- (C) CITY: Arnhem
- (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): 6824 BM
- (G) TELEPHONE: 0412 666379
- (H) TELEFAX: 0412 650592

(ii) TITLE OF INVENTION: Recombinant birnavirus vaccine

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: Patent In Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2827 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 112..2745

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC

5	CCGCCGCTGG CCGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe 5 10 15	117 Met Ser 1
10	GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro 20 25 30	165
15	GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu 35 40 45 50	213 261
20	GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCA CGG TCT Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser 55 60 65	309
25	CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu 70 75 80	357
30	GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser 85 90 95	405
35	CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAJ TAC TAC CCA ACA CAT Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His 100 105 110	453
40	CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu 115 120 125 130	501
45	CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu 135 140 145	549
50	GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys 150 155 160	597
55	GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA AAT CGA CTT GTG GCC Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu Val Ala 165 170 175	645

5	ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys 180 185 190	693
10	CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu 195 200 205 210	741
15	CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr 215 220 225	789
20	AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp 230 235 240	837
25	TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser 245 250 255	885
30	GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met 260 265 270	933
35	ATA GCT ATC TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys 275 280 285 290	981
40	CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Leu Leu Ser Met 295 300 305	1029
45	TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala 310 315 320	1077
50	GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp 325 330 335	1125
55	TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCT ATG ATC ACC TGG CCC Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro 340 345 350	1173
55	GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro 355 360 365 370	1221

5	TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val 375 380 385	1269
10	GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp 390 395 400	1317
15	AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu 405 410 415	1365
20	AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr 420 425 430	1413
25	TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn 435 440 445 450	1461
30	CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val 455 460 465	1509
35	GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln 470 475 480	1557
40	GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACA Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr 485 490 495	1605
45	CTA GTG CTT GAC CAG TGG AAC CTG ATG AGA CAG CCC AGA CCA GAC AGC Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser 500 505 510	1653
50	GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile 515 520 525 530	1701
55	GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu 535 540 545	1749
55	CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser 550 555 560	1797

5	AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser 565 570 575	1845
10	AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe 580 585 590	1893
15	TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser 595 600 605 610	1941
20	AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu 615 620 625	1989
25	AGG TTG GTA GGT GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys 630 635 640	2037
30	AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro 645 650 655	2085
35	CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu 660 665 670	2133
40	GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTA ACA TCT GAG AGC CTA Ala Phe Glu Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu 675 680 685 690	2181
45	GCC GAA CTG AAC AAG CCA GTA CCC CCC AAG CCC CCA AAT GTC AAC AGA Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg 695 700 705	2229
50	CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr 710 715 720	2277
55	GGT CGG TAC AGG AAC GAA GCC GGA CTG AGT GGT CTC GTC CTT CTA GCC Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala 725 730 735	2325
55	ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala 740 745 750	2373

5	GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe 755 760 765 770	2421
10	GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala 775 780 785	2469
15	AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu 790 795 800	2517
20	GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys 805 810 815	2565
25	AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala 820 825 830	2613
30	AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser 835 840 845 850	2661
35	AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys 855 860 865	2709
40	ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg 870 875	2755
45	GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT GCAGGGGGCCC CC	2815 2827

(2) INFORMATION FOR SEQ ID NO: 2:

50 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 878 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala			
1	5	10	15
Ala Phe Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu			
	20	25	30
Ile Pro Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser			
10	35	40	45
Arg Leu Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro			
	50	55	60
15	Arg Ser Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro		
	65	70	75
20	Asp Leu Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr		
	85	90	95
Leu Ser Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro			
25	100	105	110
Thr His Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile			
	115	120	125
30	Ala Leu Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala		
	130	135	140
Asn Glu Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg			
35	145	150	155
Asp Lys Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu			
	165	170	175
40	Val Ala Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro		
	180	185	190
Leu Lys Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile			
45	195	200	205
Thr Leu Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro			
	210	215	220
50	Leu Thr Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp		
	225	230	235
Gly Asp Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser			
55	245	250	255

Ser Ser Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly
 260 265 270
 5 Glu Met Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu
 275 280 285
 10 Leu Lys Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu
 290 295 300
 15 Ser Met Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro
 305 310 315 320
 20 Lys Ala Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn
 325 330 335
 25 Ile Trp Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr
 340 345 350
 30 Trp Pro Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly
 355 360 365
 35 Cys Pro Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg
 370 375 380
 40 Ile Val Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr
 385 390 395 400
 45 Ala Asp Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp
 405 410 415
 50 Leu Glu Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala
 420 425 430
 55 Met Tyr Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met
 435 440 445
 60 Phe Asn Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu
 450 455 460
 65 Val Val Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr
 465 470 475 480
 70 Gly Gln Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu
 485 490 495
 75 Ser Thr Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro
 500 505 510

	Asp Ser Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe		
	515	520	525
5	Lys Ile Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu		
	530	535	540
10	Val Leu Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu		
	545	550	555
	560		
	Gln Ser Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr		
	565	570	575
15	Tyr Ser Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg		
	580	585	590
20	Leu Phe Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu		
	595	600	605
	605		
	Lys Ser Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu		
	610	615	620
25	Ala Leu Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala		
	625	630	635
	640		
30	Cys Lys Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly		
	645	650	655
	655		
35	Phe Pro Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe		
	660	665	670
	670		
	Gly Glu Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu		
	675	680	685
40	Ser Leu Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val		
	690	695	700
	700		
45	Asn Arg Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu		
	705	710	715
	720		
	Lys Thr Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu		
	725	730	735
50	Leu Ala Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala		
	740	745	750
	750		
55	Glu Ala Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp		
	755	760	765

Trp Phe Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp
 770 775 780
 5 Ile Ala Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala
 785 790 795 800
 10 Leu Glu Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu
 805 810 815
 15 Val Lys Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu
 820 825 830
 20 Pro Ala Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly
 835 840 845
 25 Thr Ser Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala
 850 855 860
 Val Lys Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
 25 865 870 875

30 (2) INFORMATION FOR SEQ ID NO: 3:

35 (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 3261 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

45 (ix) FEATURE:

45 (A) NAME/KEY: CDS
 (B) LOCATION: 97..531

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

50 GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC 60
 CAGGATGGGA CTCCTCCTTC TACAAACGCTA TCATTG ATG GTT AGT AGA GAT CAG 114
 Met Val Ser Arg Asp Gln
 55 1 5

5	ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp 10 15 20	162
10	TGT TCC GTT CAT ACG GAG CCT TCT GAT GCC AAC AAC CGG ACC GGC GTC Cys Ser Val His Thr Glu Pro Ser Asp Ala Asn Asn Arg Thr Gly Val 25 30 35	210
15	CAT TCC GGA CGA CAC CCT GGA GAA GCA CAC TCT CAG GTC AGA GAC CTC His Ser Gly Arg His Pro Gly Glu Ala His Ser Gln Val Arg Asp Leu 40 45 50	258
20	GAC CTA CAA TTT GAC TGT GGG GGA CAC AGG GTC AGG GCT AAT TGT CTT Asp Leu Gln Phe Asp Cys Gly Gly His Arg Val Arg Ala Asn Cys Leu 55 60 65 70	306
25	TTT CCC TGG ATT CCC TGG CTC AAT TGT GGG TGC TCA CTA CAC ACT GCA Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly Cys Ser Leu His Thr Ala 75 80 85	354
30	GGG CAA TGG GAA CTA CAA GTT CGA TCA GAT GCT CCT GAC TGC CCA GAA Gly Gln Trp Glu Leu Gln Val Arg Ser Asp Ala Pro Asp Cys Pro Glu 90 95 100	402
35	CCT ACC GGC CAG TTA CAA CTA CTG CAG GCT AGT GAG TCG GAG TCT CAC Pro Thr Gly Gln Leu Gln Leu Gln Ala Ser Glu Ser Glu Ser His 105 110 115	450
40	AGT GAG GTC AAG CAC ACT TCC TGG TGG CGT TTA TGC ACT AAA CGG CAC Ser Glu Val Lys His Thr Ser Trp Trp Arg Leu Cys Thr Lys Arg His 120 125 130	498
45	CAT AAA CGC CGT GAC CTT CCA AGG AAG CCT GAG TGAACTGACA GATGTTAGCT His Lys Arg Arg Asp Leu Pro Arg Lys Pro Glu 135 140 145	551
50	ACAATGGTT GATGTCTGCA ACAGCCAACA TCAACGACAA AATTGGAAC GTCTAGTAG GGGAAGGGGT CACCGTCCTC AGCTTACCCA CATCATATGA TCTTGGGTAT GTGAGGCTTG GTGACCCCAT TCCCGCAATA GGGCTTGACC CAAAAATGGT AGCCACATGT GACAGCAGTG ACAGGCCAG AGTCTACACC ATAAC TGCAAG CCGATGATTA CCAATTCTCA TCACAGTACC AACCAGGTGG GGTAACAATC ACAC TGTCT CAGCCAACAT TGATGCCATC ACAAGCCTCA GCGTTGGGGG AGAGCTCGTG TTTCAAACAA GCGTCCACGG CCTTGTACTG GGCGCCACCA	611 671 731 791 851 911

	TCTACCTCAT AGGCTTGAT GGGACAACGG TAATCACCAG GGCTGTGGCC GCAAACAATG	971
5	GGCTGACGAC CGGCACCGAC AACCTTATGC CATTCAATCT TGTGATTCCA ACAAACGAGA	1031
	TAACCCAGCC AATCACATCC ATCAAACCTGG AGATAGTGAC CTCCAAAAGT GGTGGTCAGG	1091
10	CAGGGGATCA GATGTCATGG TCGGCAAGAG GGAGCCTAGC AGTGACGATC CATGGTGGCA	1151
	ACTATCCAGG GGCCCTCCGT CCCGTCACGC TAGTGGCCTA CGAAAGAGTG GCAACAGGAT	1211
15	CCGTCGTTAC GGTGCGCTGGG GTGAGCAACT TCGAGCTGAT CCCAAATCCT GAACTAGCAA	1271
	AGAACCTGGT TACAGAATAC GGCGATTG ACCCAGGAGC CATGAACCTAC ACAAAATTGA	1331
20	TACTGAGTGA GAGGGACCGT CTTGGCATCA AGACCGTCTG GCCAACAAAGG GAGTACACTG	1391
	ACTTTCGTGA ATACTTCATG GAGGTGGCCG ACCTCAACTC TCCCCTGAAG ATTGCAGGAG	1451
25	CATTGGCCTT CAAAGACATA ATCCGGGCCA TAAGGAGGAT AGCTGTGCCG GTGGTCTCCA	1511
	CATTGTTCCC ACCTGCCGCT CCCCTAGCCC ATGCAATTGG GGAAGGTGTA GACTACCTGC	1571
	TGGGCGATGA GGCACAGGCT GCTTCAGGAA CTGCTCGAGC CGCGTCAGGA AAAGCAAGAG	1631
30	CTGCCTCAGG CCGCATAAGG CAGCTGACTC TCGCCGCCGA CAAGGGGTAC GAGGTAGTCG	1691
	CGAATCTATT CCAGGTGCC CAGAATCCCG TAGTCGACGG GATTCTTGCT TCACCTGGGG	1751
35	TACTCCGCGG TGCACACAAAC CTCGACTGCG TGTTAAGAGA GGGTGCCACG CTATTCCCTG	1811
	TGGTTATTAC GACAGTGGAA GACGCCATGA CACCCAAAGC ATTGAACAGC AAAATGTTT	1871
40	CTGTCATTGA AGGCGTGGCA GAAGACCTCC AACCTCCATC TCAAAGAGGA TCCTTCATAC	1931
	GAACTCTCTC TGGACACAGA GTCTATGGAT ATGCTCCAGA TGGGGTACTT CCACTGGAGA	1991
45	CTGGGAGAGA CTACACCGTT GTCCCAATAG ATGATGTCTG GGACGACAGC ATTATGCTGT	2051
	CCAAAGATCC CATACTCCT ATTGTGGAA ACAGTGGAAA TCTAGCCATA GCTTACATGG	2111
	ATGTGTTTCG ACCCAAAGTC CCAATCCATG TGGCTATGAC GGGAGCCCTC AATGCTTGTG	2171
50	GCGAGATTGA GAAAGTAAGC TTTAGAAGCA CCAAGCTCGC CACTGCACAC CGACTTGGCC	2231
	TTAGGTTGGC TGGTCCCGGA GCATTCGATG TAAACACCGG GCCCAAATGG GCAACGTTCA	2291
55	TCAAACGTTT CCCTCACAAT CCACGCGACT GGGACAGGCT CCCCTACCTC AACCTACCAT	2351

ACCTTCCACC	CAATGCAGGA	CGCCAGTACC	ACCTGCCAT	GGCTGCATCA	GAGTTCAAAG	2411	
5	AGACCCCCGA	ACTCGAGAGT	GCCGTCAGAG	CAATGGAAGC	AGCAGCCAAC	GTGGACCCAC	2471
	TATTCCAATC	TGCACTCAGT	GTGTTCATGT	GGCTGGAAGA	GAATGGGATT	GTGACTGACA	2531
10	TGGCCAACCTT	CGCACTCAGC	GACCCGAACG	CCCATCGGAT	GCGAAATTTT	CTTGCAAACG	2591
	CACCAACAAGC	AGGCAGCAAG	TCGCAAAGGG	CCAAGTACGG	GACAGCAGGC	TACGGAGTGG	2651
15	AGGCTCGGGG	CCCCACACCA	GAGGAAGCAC	AGAGGGAAAA	AGACACACGG	ATCTCAAAGA	2711
	AGATGGAGAC	CATGGGCATC	TACTTGCAA	CACCAGAATG	GGTAGCACTC	AATGGGCACC	2771
20	GAGGGCCAAG	CCCCGGCCAG	CTAAAGTACT	GGCAGAACAC	ACGAGAAATA	CCGGACCCAA	2831
	ACGAGGACTA	TCTAGACTAC	GTGCATGCAG	AGAAGAGCCG	GTTGGCATCA	GAAGAACAAA	2891
25	TCCTAAGGGC	AGCTACGTCG	ATCTACGGGG	CTCCAGGACA	GGCAGAGCCA	CCCCAAGCTT	2951
	TCATAGACGA	AGTTGCCAAA	GTCTATGAAA	TCAACCATGG	ACGTGGCCA	AACCAAGAAC	3011
	AGATGAAAGA	TCTGCTCTTG	ACTGCGATGG	AGATGAAGCA	TCGCAATCCC	AGGCAGGGCTC	3071
30	TACCAAAGCC	CAAGCCAAAA	CCCAATGCTC	CAACACAGAG	ACCCCTGGT	CGGCTGGGCC	3131
	GCTGGATCAG	GACCGTCTCT	GATGAGGACC	TTGAGTGAGG	CTCCTGGGAG	TCTCCCGACA	3191
35	CCACCCGCGC	AGGTGTGGAC	ACCAATTGG	CCTTACAACA	TCCCAAATTG	GATCCGTTCG	3251
	CGGGTCCCCT						3261
40							

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 145 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met	Val	Ser	Arg	Asp	Gln	Thr	Asn	Asp	Arg	Ser	Asp	Asp	Lys	Pro	Ala
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

55	1	5	10	15
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Arg Ser Asn Pro Thr Asp Cys Ser Val His Thr Glu Pro Ser Asp Ala
 20 25 30
 5 Asn Asn Arg Thr Gly Val His Ser Gly Arg His Pro Gly Glu Ala His
 35 40 45
 10 Ser Gln Val Arg Asp Leu Asp Leu Gln Phe Asp Cys Gly Gly His Arg
 50 55 60
 15 Val Arg Ala Asn Cys Leu Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly
 65 70 75 80
 20 Cys Ser Leu His Thr Ala Gly Gln Trp Glu Leu Gln Val Arg Ser Asp
 85 90 95
 25 Ala Pro Asp Cys Pro Glu Pro Thr Gly Gln Leu Gln Leu Leu Gln Ala
 100 105 110
 30 Ser Glu Ser Glu Ser His Ser Glu Val Lys His Thr Ser Trp Trp Arg
 115 120 125
 35 Leu Cys Thr Lys Arg His His Lys Arg Arg Asp Leu Pro Arg Lys Pro
 130 135 140
 40 Glu
 145

35 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 3261 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

50 (A) NAME/KEY: CDS
 (B) LOCATION: 131..3166

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

55 GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GCCCTTGTTC

60

	CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTGATGG TTAGTAGAGA TCAGACAAAC	120
5	GATCGCAGCG ATG ACA AAC CTG CAA GAT CAA ACC CAA CAG ATT GTT CCG Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro	169
	1 5 10	
10	TTC ATA CGG AGC CTT CTG ATG CCA ACA ACC GGA CCG GCG TCC ATT CCG Phe Ile Arg Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro	217
	15 20 25	
15	GAC GAC ACC CTG GAG AAG CAC ACT CTC AGG TCA GAG ACC TCG ACC TAC Asp Asp Thr Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr	265
	30 35 40 45	
20	AAT TTG ACT GTG GGG GAC ACA GGG TCA GGG CTA ATT GTC TTT TTC CCT Asn Leu Thr Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro	313
	50 55 60	
25	GGA TTC CCT GGC TCA ATT GTG GGT GCT CAC TAC ACA CTG CAG GGC AAT Gly Phe Pro Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn	361
	65 70 75	
30	GGG AAC TAC AAG TTC GAT CAG ATG CTC CTG ACT GCC CAG AAC CTA CCG Gly Asn Tyr Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro	409
	80 85 90	
35	GCC AGT TAC AAC TAC TGC AGG CTA GTG AGT CGG AGT CTC ACA GTG AGG Ala Ser Tyr Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg	457
	95 100 105	
40	TCA AGC ACA CTT CCT GGT GGC GTT TAT GCA CTA AAC GGC ACC ATA AAC Ser Ser Thr Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn	505
	110 115 120 125	
45	GCC GTG ACC TTC CAA GGA AGC CTG AGT GAA CTG ACA GAT GTT AGC TAC Ala Val Thr Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr	553
	130 135 140	
50	AAT GGG TTG ATG TCT GCA ACA GCC AAC ATC AAC GAC AAA ATT GGG AAC Asn Gly Leu Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn	601
	145 150 155	
55	GTC CTA GTA GGG GAA GGG GTC ACC GTC CTC AGC TTA CCC ACA TCA TAT Val Leu Val Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr	649
	160 165 170	
	GAT CTT GGG TAT GTG AGG CTT GGT GAC CCC ATT CCC GCA ATA GGG CTT	697

	Asp Leu Gly Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu		
	175	180	185
5	GAC CCA AAA ATG GTA GCC ACA TGT GAC AGC AGT GAC AGG CCC AGA GTC		745
	Asp Pro Lys Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val		
	190	195	200
10	TAC ACC ATA ACT GCA GCC GAT GAT TAC CAA TTC TCA TCA CAG TAC CAA		793
	Tyr Thr Ile Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln		
	210	215	220
15	CCA GGT GGG GTA ACA ATC ACA CTG TTC TCA GCC AAC ATT GAT GCC ATC		841
	Pro Gly Gly Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile		
	225	230	235
20	ACA AGC CTC AGC GTT GGG GGA GAG CTC GTG TTT CAA ACA AGC GTC CAC		889
	Thr Ser Leu Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His		
	240	245	250
25	GGC CTT GTA CTG GGC GCC ACC ATC TAC CTC ATA GGC TTT GAT GGG ACA		937
	Gly Leu Val Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr		
	255	260	265
30	ACG GTA ATC ACC AGG GCT GTG GCC GCA AAC AAT GGG CTG ACG ACC GGC		985
	Thr Val Ile Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly		
	270	275	280
	285		
35	ACC GAC AAC CTT ATG CCA TTC AAT CTT GTG ATT CCA ACA AAC GAG ATA		1033
	Thr Asp Asn Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile		
	290	295	300
40	ACC CAG CCA ATC ACA TCC ATC AAA CTG GAG ATA GTG ACC TCC AAA AGT		1081
	Thr Gln Pro Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser		
	305	310	315
45	GGT GGT CAG GCA GGG GAT CAG ATG TCA TGG TCG GCA AGA GGG AGC CTA		1129
	Gly Gly Gln Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu		
	320	325	330
50	GCA GTG ACG ATC CAT GGT GGC AAC TAT CCA GGG GCC CTC CGT CCC GTC		1177
	Ala Val Thr Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val		
	335	340	345
55	ACG CTA GTG GCC TAC GAA AGA GTG GCA ACA GGA TCC GTC GTT ACG GTC		1225
	Thr Leu Val Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val		
	350	355	360
	365		
	GCT GGG GTG AGC AAC TTC GAG CTG ATC CCA AAT CCT GAA CTA GCA AAG		1273

	Ala Gly Val Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys		
	370	375	380
5	AAC CTG GTT ACA GAA TAC GGC CGA TTT GAC CCA GGA GCC ATG AAC TAC Asn Leu Val Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr		1321
	385	390	395
10	ACA AAA TTG ATA CTG AGT GAG AGG GAC CGT CTT GGC ATC AAG ACC GTC Thr Lys Leu Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val		1369
	400	405	410
15	TGG CCA ACA AGG GAG TAC ACT GAC TTT CGT GAA TAC TTC ATG GAG GTG Trp Pro Thr Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val		1417
	415	420	425
20	GCC GAC CTC AAC TCT CCC CTG AAG ATT GCA GGA GCA TTC GGC TTC AAA Ala Asp Leu Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys		1465
	430	435	440
	445		
25	GAC ATA ATC CGG GCC ATA AGG AGG ATA GCT GTG CCG GTG GTC TCC ACA Asp Ile Ile Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr		1513
	450	455	460
30	TTG TTC CCA CCT GCC GCT CCC CTA GCC CAT GCA ATT GGG GAA GGT GTA Leu Phe Pro Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val		1561
	465	470	475
35	GAC TAC CTG CTG GGC GAT GAG GCA CAG GCT GCT TCA GGA ACT GCT CGA Asp Tyr Leu Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg		1609
	480	485	490
40	GCC GCG TCA GGA AAA GCA AGA GCT GCC TCA GGC CGC ATA AGG CAG CTG Ala Ala Ser Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu		1657
	495	500	505
45	ACT CTC GCC GCC GAC AAG GGG TAC GAG GTA GTC GCG AAT CTA TTC CAG Thr Leu Ala Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln		1705
	510	515	520
	525		
50	GTG CCC CAG AAT CCC GTA GTC GAC GGG ATT CTT GCT TCA CCT GGG GTA Val Pro Gln Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val		1753
	530	535	540
55	CTC CGC GGT GCA CAC AAC CTC GAC TGC GTG TTA AGA GAG GGT GCC ACG Leu Arg Gly Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr		1801
	545	550	555
	CTA TTC CCT GTG GTT ATT ACG ACA GTG GAA GAC GGC ATG ACA CCC AAA		1849

	Leu Phe Pro Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys		
	560	565	570
5	GCA TTG AAC AGC AAA ATG TTT GCT GTC ATT GAA GGC GTG CGA GAA GAC		1897
	Ala Leu Asn Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp		
	575	580	585
10	CTC CAA CCT CCA TCT CAA AGA GGA TCC TTC ATA CGA ACT CTC TCT GGA		1945
	Leu Gln Pro Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly		
	590	595	600
	605		
15	CAC AGA GTC TAT GGA TAT GCT CCA GAT GGG GTA CTT CCA CTG GAG ACT		1993
	His Arg Val Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr		
	610	615	620
20	GGG AGA GAC TAC ACC GTT GTC CCA ATA GAT GAT GTC TGG GAC GAC AGC		2041
	Gly Arg Asp Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser		
	625	630	635
25	ATT ATG CTG TCC AAA GAT CCC ATA CCT CCT ATT GTG GGA AAC AGT GGA		2089
	Ile Met Leu Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly		
	640	645	650
30	AAT CTA GCC ATA GCT TAC ATG GAT GTG TTT CGA CCC AAA GTC CCA ATC		2137
	Asn Leu Ala Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile		
	655	660	665
35	CAT GTG GCT ATG ACG GGA GCC CTC AAT GCT TGT GGC GAG ATT GAG AAA		2185
	His Val Ala Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys		
	670	675	680
	685		
40	GTA AGC TTT AGA AGC ACC AAG CTC GCC ACT GCA CAC CGA CTT GGC CTT		2233
	Val Ser Phe Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu		
	690	695	700
45	AGG TTG GCT GGT CCC GGA GCA TTC GAT GTA AAC ACC GGG CCC AAC TGG		2281
	Arg Leu Ala Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp		
	705	710	715
50	GCA ACG TTC ATC AAA CGT TTC CCT CAC AAT CCA CGC GAC TGG GAC AGG		2329
	Ala Thr Phe Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg		
	720	725	730
55	CTC CCC TAC CTC AAC CTA CCA TAC CTT CCA CCC AAT GCA GGA CGC CAG		2377
	Leu Pro Tyr Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln		
	735	740	745
	TAC CAC CTT GCC ATG GCT GCA TCA GAG TTC AAA GAG ACC CCC GAA CTC		2425

	Tyr His Leu Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu		
750	755	760	765
5	GAG AGT GCC GTC AGA GCA ATG GAA GCA GCA AAC GTG GAC CCA CTA		2473
	Glu Ser Ala Val Arg Ala Met Glu Ala Ala Asn Val Asp Pro Leu		
	770	775	780
10	TTC CAA TCT GCA CTC AGT GTG TTC ATG TGG CTG GAA GAG AAT GGG ATT		2521
	Phe Gln Ser Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile		
	785	790	795
15	GTG ACT GAC ATG GCC AAC TTC GCA CTC AGC GAC CCG AAC GCC CAT CGG		2569
	Val Thr Asp Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg		
	800	805	810
20	ATG CGA AAT TTT CTT GCA AAC GCA CCA CAA GCA GGC AGC AAG TCG CAA		2617
	Met Arg Asn Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln		
	815	820	825
25	AGG GCC AAG TAC GGG ACA GCA GGC TAC GGA GTG GAG GCT CGG GGC CCC		2665
	Arg Ala Lys Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro		
	830	835	840
	845		
30	ACA CCA GAG GAA GCA CAG AGG GAA AAA GAC ACA CGG ATC TCA AAG AAG		2713
	Thr Pro Glu Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys		
	850	855	860
35	ATG GAG ACC ATG GGC ATC TAC TTT GCA ACA CCA GAA TGG GTA GCA CTC		2761
	Met Glu Thr Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu		
	865	870	875
	AAT GGG CAC CGA GGG CCA AGC CCC GGC CAG CTA AAG TAC TGG CAG AAC		2809
	Asn Gly His Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn		
	880	885	890
40	ACA CGA GAA ATA CCG GAC CCA AAC GAG GAC TAT CTA GAC TAC GTG CAT		2857
	Thr Arg Glu Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His		
	895	900	905
45	GCA GAG AAG ACC CGG TTG GCA TCA GAA GAA CAA ATC CTA AGG GCA GCT		2905
	Ala Glu Lys Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala		
	910	915	920
	925		
50	ACG TCG ATC TAC GGG GCT CCA GGA CAG GCA GAG CCA CCC CAA GCT TTC		2953
	Thr Ser Ile Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe		
	930	935	940
55	ATA GAC GAA GTT GCC AAA GTC TAT GAA ATC AAC CAT GGA CGT GGC CCA		3001

Ile Asp Glu Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro
 945 950 955

5 AAC CAA GAA CAG ATG AAA GAT CTG CTC TTG ACT GCG ATG GAG ATG AAG 3049
 Asn Gln Glu Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys
 960 965 970

10 CAT CGC AAT CCC AGG CGG GCT CTA CCA AAG CCC AAG CCA AAA CCC AAT 3097
 His Arg Asn Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn
 975 980 985

15 GCT CCA ACA CAG AGA CCC CCT GGT CGG CTG GGC CGC TGG ATC AGG ACC 3145
 Ala Pro Thr Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr
 990 995 1000 1005

20 GTC TCT GAT GAG GAC CTT GAG TGAGGCTCCT GGGAGTCTCC CGACACCACC 3196
 Val Ser Asp Glu Asp Leu Glu
 1010

25 CGCGCAGGTG TGGACACCAA TTCGGCCTTA CAACATCCCA AATTGGATCC GTTCGCGGGT 3256

CCCCT 3261

30 (2) INFORMATION FOR SEQ ID NO: 6:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1012 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

45 Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg
 1 5 10 15

50 Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
 20 25 30

55 Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
 35 40 45

60 Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
 50 55 60

65 Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr

	65	70	75	80
5	Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr			
	85	90		95
10	Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr			
	100	105		110
15	Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr			
	115	120		125
20	Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu			
	130	135	140	
25	Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val			
	145	150	155	160
30	Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly			
	165	170		175
35	Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys			
	180	185		190
40	Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile			
	195	200	205	
45	Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly			
	210	215	220	
50	Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu			
	225	230	235	240
	Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His Gly Leu Val			
	245	250		255
	Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile			
	260	265		270
	Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn			
	275	280	285	
	Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro			
	290	295	300	
	Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln			
	305	310	315	320
55	Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu Ala Val Thr			

	325	330	335
5	Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val		
	340	345	350
10	Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val		
	355	360	365
15	Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val		
	370	375	380
20	Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu		
	385	390	395
25	Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr		
	405	410	415
30	Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu		
	420	425	430
35	Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile		
	435	440	445
40	Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr Leu Phe Pro		
	450	455	460
45	Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val Asp Tyr Leu		
	465	470	475
50	Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg Ala Ala Ser		
	485	490	495
55	Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu Thr Leu Ala		
	500	505	510
60	Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln Val Pro Gln		
	515	520	525
65	Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val Leu Arg Gly		
	530	535	540
70	Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr Leu Phe Pro		
	545	550	555
75	Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys Ala Leu Asn		
	565	570	575
80	Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp Leu Gln Pro		

	580	585	590
5	Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly His Arg Val		
	595	600	605
	Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr Gly Arg Asp		
10	610	615	620
	Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser Ile Met Leu		
	625	630	635
15	Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly Asn Leu Ala		
	645	650	655
	Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile His Val Ala		
20	660	665	670
	Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys Val Ser Phe		
	675	680	685
25	Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu Arg Leu Ala		
	690	695	700
	Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp Ala Thr Phe		
30	705	710	715
	Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg Leu Pro Tyr		
	725	730	735
35	Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln Tyr His Leu		
	740	745	750
	Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala		
40	755	760	765
	Val Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser		
	770	775	780
45	Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp		
	785	790	795
	Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn		
50	805	810	815
	Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys		
	820	825	830
55	Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu		

	835	840	845
5	Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr 850	855	860
10	Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His 865	870	875
	Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn Thr Arg Glu 885	890	895
15	Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys 900	905	910
20	Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile 915	920	925
	Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu 930	935	940
25	Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu 945	950	955
	960		
30	Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn 965	970	975
	980		
35	Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn Ala Pro Thr 985	990	
	995		
40	Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr Val Ser Asp 1000	1005	
	1010		

45

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 3261 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION:97..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

5

GGATACGATC	GGTCTGACCC	CGGGGGAGTC	ACCCGGGGAC	AGGCCGTCAA	GGCCTTGTTC	60											
10	CAGGATGGGA	CTCCTCCTTC	TACAACGCTA	TCATTC	GAA	GTT	AGT	TGA	GAT	CTG	114						
						Glu	Val	Ser	*	Asp	Leu						
						1				5							
15	ACA	AAC	GAT	CGC	AGC	GAT	GAC	AAA	CCT	GCA	AGA	TCA	AAC	CCA	ACA	GAT	162
	Thr	Asn	Asp	Arg	Ser	Asp	Asp	Lys	Pro	Ala	Arg	Ser	Asn	Pro	Thr	Asp	
	10								15						20		

20 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 2827 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

35 (A) NAME/KEY: CDS
 (B) LOCATION:112..2745

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

40	GGATACGATG	GGTCTGACCC	TCTGGGAGTC	ACGAATTAAC	GTGGCTACTA	GGGGCGATAC	60										
	CCGCCGCTGG	CTGCCACGTT	AGTGGCTCCT	CTTCTTGATG	ATTCTGCCAC	C	ATG	AGT	117								
45							Met	Ser									
							1										
50	GAC	ATT	TTC	AAC	AGT	CCA	CAG	GCG	CGA	AGC	ACG	ATC	TCA	GCA	GCG	TTC	165
	Asp	Ile	Phe	Asn	Ser	Pro	Gln	Ala	Arg	Ser	Thr	Ile	Ser	Ala	Ala	Phe	
	5					10						15					
	GGC	ATA	AAG	CCT	ACT	GCT	GGA	CAA	GAC	GTG	GAA	GAA	CTC	TTG	ATC	CCT	213

	Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro		
20	25	30	
5	AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu		261
35	40	45	50
10	GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCG CGG TCT Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser		309
55	60	65	
15	CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu		357
70	75	80	
20	GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser		405
85	90	95	
25	CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAG TAC TAC CCA ACA CAT Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His		453
100	105	110	
30	CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu		501
115	120	125	130
35	CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu		549
135	140	145	
40	GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys		597
150	155	160	
45	GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA ACT CGA CTT GTG GCC Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Thr Arg Leu Val Ala		645
165	170	175	
50	ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys		693
180	185	190	
55	CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu		741
195	200	205	210
55	CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA		789

	Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr		
	215	220	225
5	AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC		837
	Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp		
	230	235	240
10	TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT		885
	Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser		
	245	250	255
15	GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG		933
	Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met		
	260	265	270
20	ATA GCT ATA TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG		981
	Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys		
	275	280	285
	290		
25	CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG		1029
	Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met		
	295	300	305
30	TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT		1077
	Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala		
	310	315	320
35	GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG		1125
	Glu Arg Tyr Asp Lys Ser Thr Trp Leu Th: Lys Thr Arg Asn Ile Trp		
	325	330	335
40	TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCC ATG ATC ACC TGG CCC		1173
	Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro		
	340	345	350
45	GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA		1221
	Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro		
	355	360	365
	370		
50	TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC		1269
	Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Leu Asn Arg Ile Val		
	375	380	385
55	GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC		1317
	Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp		
	390	395	400
	400		
	AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG		1365

	Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu		
	405	410	415
5	AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC		1413
	Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr		
	420	425	430
10	TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT		1461
	Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn		
	435	440	445
	450		
15	CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG		1509
	Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val		
	455	460	465
20	GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA		1557
	Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln		
	470	475	480
25	GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACG		1605
	Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr		
	485	490	495
30	CTA GTG CTT GAC CAG TGG AAC TTG ATG AGA CAG CCC AGA CCA GAC AGC		1653
	Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser		
	500	505	510
35	GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT		1701
	Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile		
	515	520	525
	530		
40	GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC		1749
	Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu		
	535	540	545
45	CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC		1797
	Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Gln Ser		
	550	555	560
50	AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC		1845
	Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser		
	565	570	575
55	AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT		1893
	Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe		
	580	585	590
	TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC		1941

	Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser		
595	600	605	610
5	AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG		1989
	Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu		
	615	620	625
10	AGG TTG GTA GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG		2037
	Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys		
	630	635	640
15	AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA		2085
	Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro		
	645	650	655
20	CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG		2133
	Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu		
	660	665	670
25	GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTC ACA TCT GAG AGC CTA		2181
	Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu		
	675	680	685
30	GCC GAA CTG AAC AAG CCA GTC CCC CCC AAG CCC CCA AAT GTC AAC AGA		2229
	Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg		
	695	700	705
35	CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC		2277
	Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr		
	710	715	720
40	GGT CGG TAC AGG AAC GAA GCA GGA CTG AGT GGT CTC GTC CTT CTA GCC		2325
	Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala		
	725	730	735
45	ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC		2373
	Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala		
	740	745	750
50	GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC		2421
	Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe		
	755	760	765
	770		
55	GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC		2469
	Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala		
	775	780	785
	AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA		2517

Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu	790	795	800	
5				
GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG				2565
Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys				
805	810	815		
10				
AAC CCA CAG ACC GCC TCC AAC CCC GTT GGG CTC CAC CTG CCC GCC				2613
Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala				
820	825	830		
15				
AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC				2661
Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser				
835	840	845	850	
20				
AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA				2709
Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys				
855	860	865		
25				
ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT				2755
Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg				
870	875			
30				
GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT				2815
GCGGGGGCCC CC				2827
35				

Claims

- 40 1. A birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome, **characterised in that** the mutation comprises:
 - (i) a substitution of at least two nucleotides of the start codon of the VP5 gene, and
 - (ii) a stop codon in each of the three open reading frames in the 5'-end of the VP5 gene.
- 45 2. A birnavirus mutant according to claim 2, **characterised in that** the birnavirus is infectious bursal disease virus (IBDV).
- 50 3. A birnavirus mutant according to claims 1-2, **characterised in that** the mutation is in the genome of a virulent field virus.
- 55 4. A birnavirus mutant according to claim 2, **characterised in that** the mutation is in the genome of a vaccine strain, preferably in vaccine strain D78.
- 55 5. A birnavirus mutant according to claims 2-4, **characterised in that** the mutant has a mutated start codon and three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.
- 55 6. A birnavirus mutant according to claims 2-5, **characterised in that** the IBDV expresses a chimeric VP2 protein

comprising virus neutralising epitopes of different antigenic IBDV types.

7. A vaccine against a birnavirus infection in animals, **characterised in that** it comprises a birnavirus mutant according to claims 1-6 and a pharmaceutically acceptable carrier.
8. A method for the attenuation of virulence of a birnavirus in an animal, comprising the step of introducing a mutation in the VP5 gene as a result of which the birnavirus is not able to produce a VP5 protein.
9. A method according to claim 9, wherein the mutation is a substitution.
10. A method according to claims 8-9, wherein the birnavirus is infectious bursal disease virus (IBDV).
11. A method according to claims 8-10, wherein the mutation is in the genome of a virulent field virus.
12. A method according to claims 8-11, wherein the mutation comprises a substitution of at least two nucleotides of the start codon of the VP5 gene.
13. A method according to claim 12 wherein the mutation comprises additionally one or more stop codons in the 5'-end of the VP5 gene.
14. A method according to claim 13, wherein the mutation comprises a stop codon in each of the three open reading frames.
15. A method according to claim 14, wherein the mutation is in the start codon and comprises three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.

Patentansprüche

30. 1. Eine Birnavirus Mutante, die aufgrund einer Mutation im VP5 Gen des Birnavirus Genoms kein natives VP5 Protein produzieren kann, **dadurch gekennzeichnet, dass** die Mutation:
 - (i) eine Substitution von mindestens zwei Nukleotiden des Startcodons des VP5 Gens, und
 - (ii) ein Stopcodon in jedem der drei offenen Leserahmen am 5'-Ende des VP5 Gens.
 umfasst.
35. 2. Eine Birnavirus Mutante gemäss Anspruch 1, **dadurch gekennzeichnet dass** das Birnavirus ein Infektiöses Bur-sitis Virus (IBDV) darstellt.
40. 3. Eine Birnavirus Mutante gemäss Ansprüchen 1-2, **dadurch gekennzeichnet, dass** die Mutation im Genom eines virulenten Feldvirus ist.
45. 4. Eine Birnavirus Mutante gemäss Anspruch 2, **dadurch gekennzeichnet, dass** die Mutation im Genom eines Impf-stoff-Stammes, vorzugsweise im Impfstoff-Stamm D78, ist.
50. 5. Eine Birnavirus Mutante gemäss Ansprüchen 2-4, **dadurch gekennzeichnet, dass** die Mutante ein mutiertes Startcodon und drei Stopcodons am 5'-Ende des VP5-Gens wie in SEQ ID No:7 dargestellt besitzt.
55. 6. Eine Birnavirus Mutante gemäss Ansprüchen 2-5, **dadurch gekennzeichnet, dass** das IBDV ein chimäres VP2 Protein exprimiert, das Virus-neutralisierende Epitope von unterschiedlichen, antigenen IBDV-Typen umfasst.
7. Ein Impfstoff gegen eine Birnavirus Infektion in Tieren, **dadurch gekennzeichnet, dass** er eine Birnavirus Mutante gemäss Ansprüchen 1-6 und einen pharmazeutisch verträglichen Träger umfasst.
8. Verfahren zur Abschwächung der Virulenz eines Birnavirus in einem Tier, welches den Schritt des Einführens einer Mutation in das VP5 Gen umfasst, als Folge dessen das Birnavirus kein VP5 Protein produzieren kann.

9. Verfahren gemäss Anspruch 8, worin die Mutation eine Substitution darstellt.
10. Verfahren gemäss Ansprüchen 8-9, worin das Birnavirus ein Infektiöses Bursitis Virus (IBDV) darstellt.
- 5 11. Verfahren gemäss Ansprüchen 8-10, worin die Mutation im Genom eines virulenten Feldvirus ist.
12. Verfahren gemäss Ansprüchen 8-11, worin die Mutation eine Substitution von mindestens zwei Nukleotiden des Startcodons des VP5 Gens umfasst.
- 10 13. Verfahren gemäss Anspruch 12, worin die Mutation zusätzlich ein oder mehrere Stopcodons am 5'-Ende des VP5-Gens umfasst.
14. Verfahren gemäss Anspruch 13, worin die Mutation in jedem der drei offenen Leserahmen ein Stopcodon umfasst.
- 15 15. Verfahren gemäss Anspruch 14, worin die Mutation im Startcodon ist und drei Stopcodons am 5'-Ende des VP5-Gens wie in SEQ ID No:7 dargestellt umfasst.

Revendications

- 20 1. Un mutant du birnavirus qui est incapable de produire une protéine VP5 native, résultant d'une mutation dans le gène VP5 du génome du birnavirus, **caractérisé en ce que** la mutation comprend :
 - i) une substitution d'au moins deux nucléotides du codon de départ du gène VP5, et
 - 25 ii) un codon d'arrêt dans chacun des trois cadres ouverts de lecture dans l'extrémité 5' du gène VP5.
2. Un mutant du birnavirus selon la revendication 1, **caractérisé en ce que** le birnavirus est le virus de la bursite infectieuse aviaire (IBDV : *Infectious Bursal Disease Virus*).
- 30 3. Un mutant du birnavirus selon la revendication 1 ou 2, **caractérisé en ce que** la mutation est dans le génome d'un virus sauvage virulent.
4. Un mutant du birnavirus selon la revendication 2, **caractérisé en ce que** la mutation est dans le génome d'une souche de vaccin, de préférence dans la souche de vaccin D78.
- 35 5. Un mutant du birnavirus selon l'une des revendications 2 à 4, **caractérisé en ce que** le mutant a un codon de départ ayant subi une mutation et les trois codons d'arrêt dans l'extrémité 5' du gène VP5 tel que représenté dans la SEQ ID n° 7.
- 40 6. Un mutant du birnavirus selon l'une des revendications 2 à 5, **caractérisé en ce que** l'IBDV exprime une protéine chimère VP2 comprenant des épitopes neutralisant des virus de différents types antigènes d'IBDV.
7. Un vaccin contre une infection par le birnavirus chez des animaux, **caractérisé en ce qu'il** comprend un mutant du birnavirus selon l'une des revendications 1 à 6 et un support pharmaceutiquement acceptable.
- 45 8. Un procédé pour atténuer la virulence d'un birnavirus chez un animal, comprenant l'étape consistant à introduire une mutation dans le gène VP5 pour que le birnavirus soit incapable de produire une protéine VP5.
9. Un procédé selon la revendication 9, dans lequel la mutation est une substitution.
- 50 10. Un procédé selon la revendication 8 ou 9, dans lequel le birnavirus est le virus de la bursite infectieuse aviaire (IBDV).
11. Un procédé selon l'une des revendications 8 à 10, dans lequel la mutation est dans le génome d'un virus sauvage virulent.
- 55 12. Un procédé selon l'une des revendications 8 à 11, dans lequel la mutation comprend une substitution d'au moins deux nucléotides du codon de départ du gène VP5.

13. Un procédé selon la revendication 12, dans lequel la mutation comprend en outre un ou plusieurs codons d'arrêt dans l'extrémité 5' du gène VP5.
- 5 14. Un procédé selon la revendication 13, dans lequel la mutation comprend un codon d'arrêt dans chacun des trois cadres ouverts de lecture.
15. Un procédé selon la revendication 14, dans lequel la mutation est située dans le codon de départ et comprend trois codons d'arrêt dans l'extrémité 5' du gène VP5 tel que représenté dans la SEQ ID n° 7.

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Figure 2

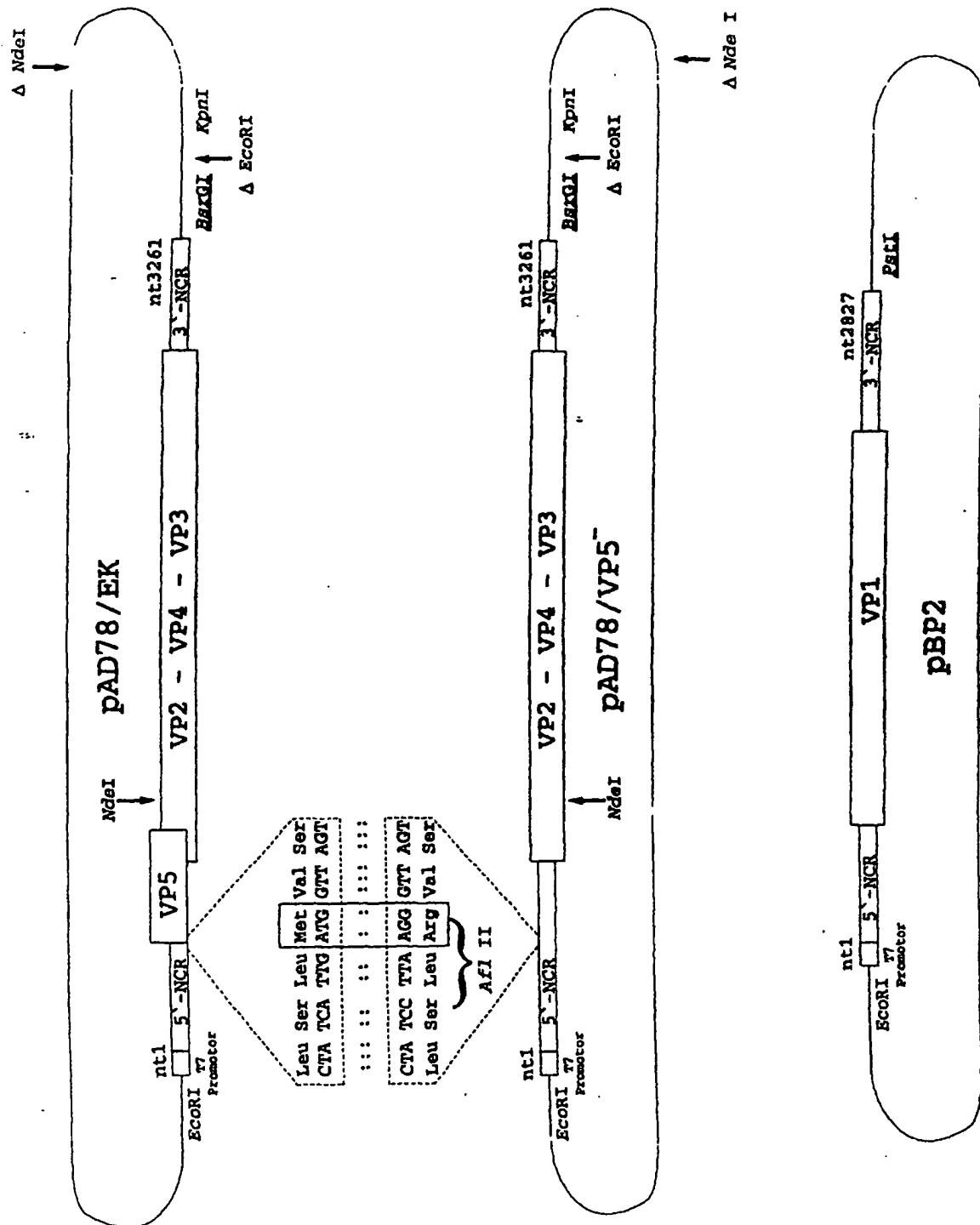


Figure 3

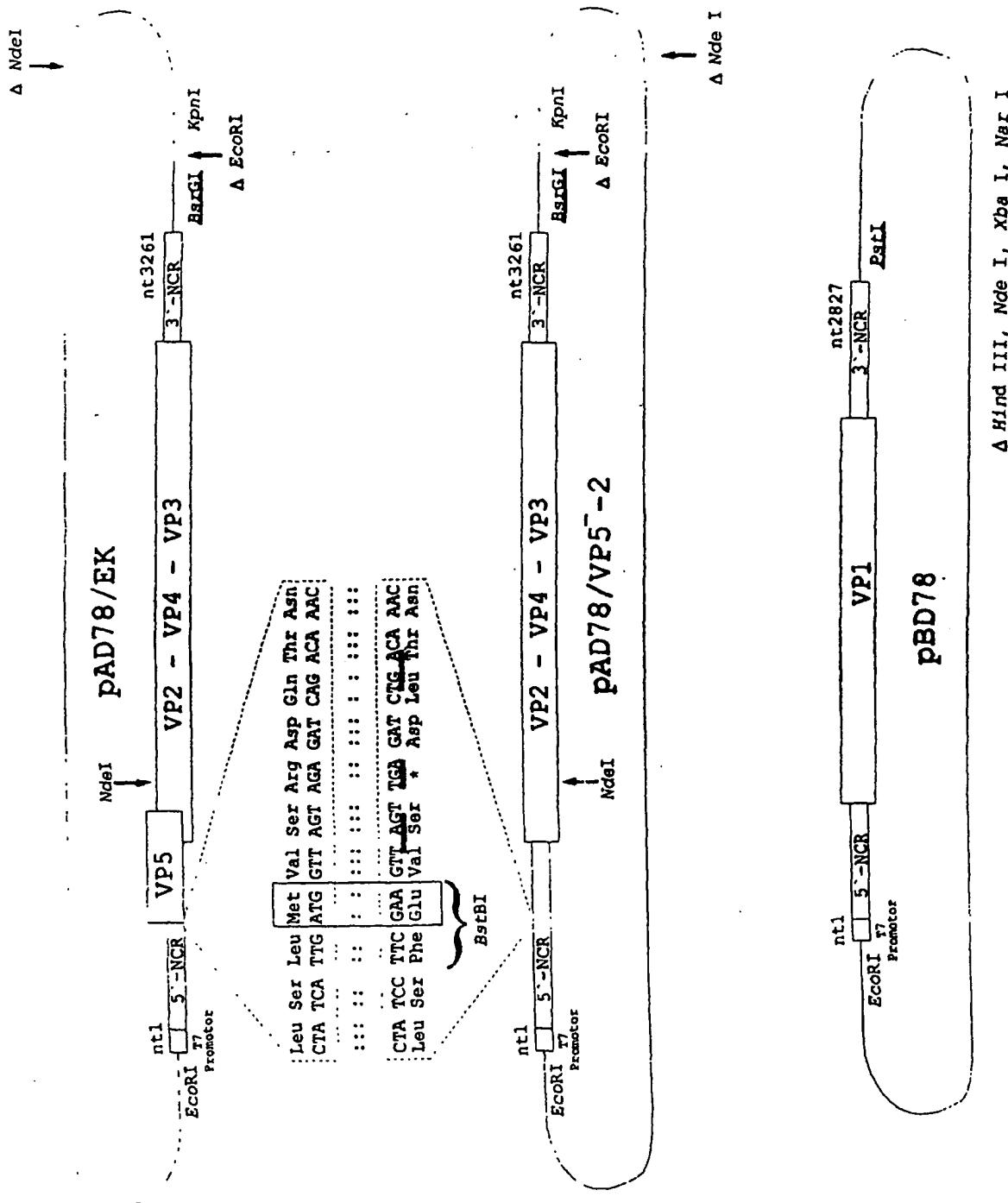


Figure 4

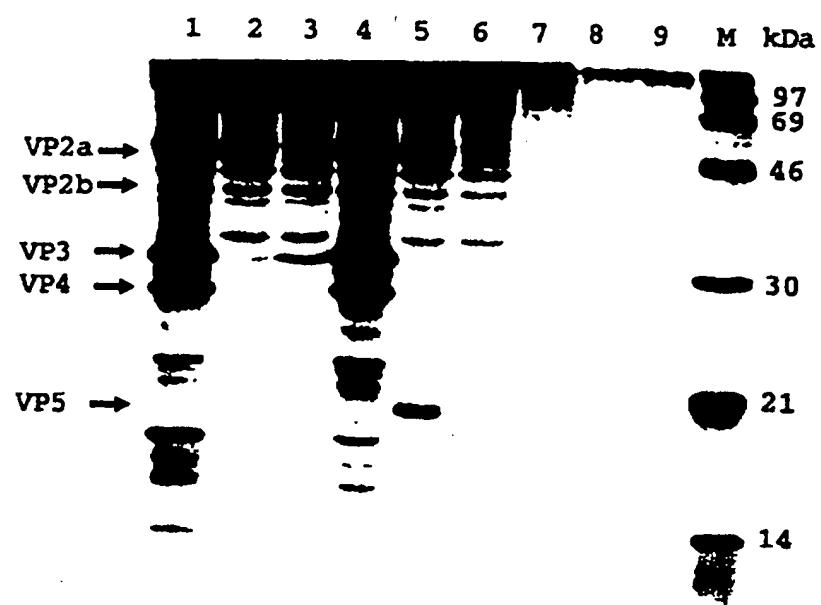
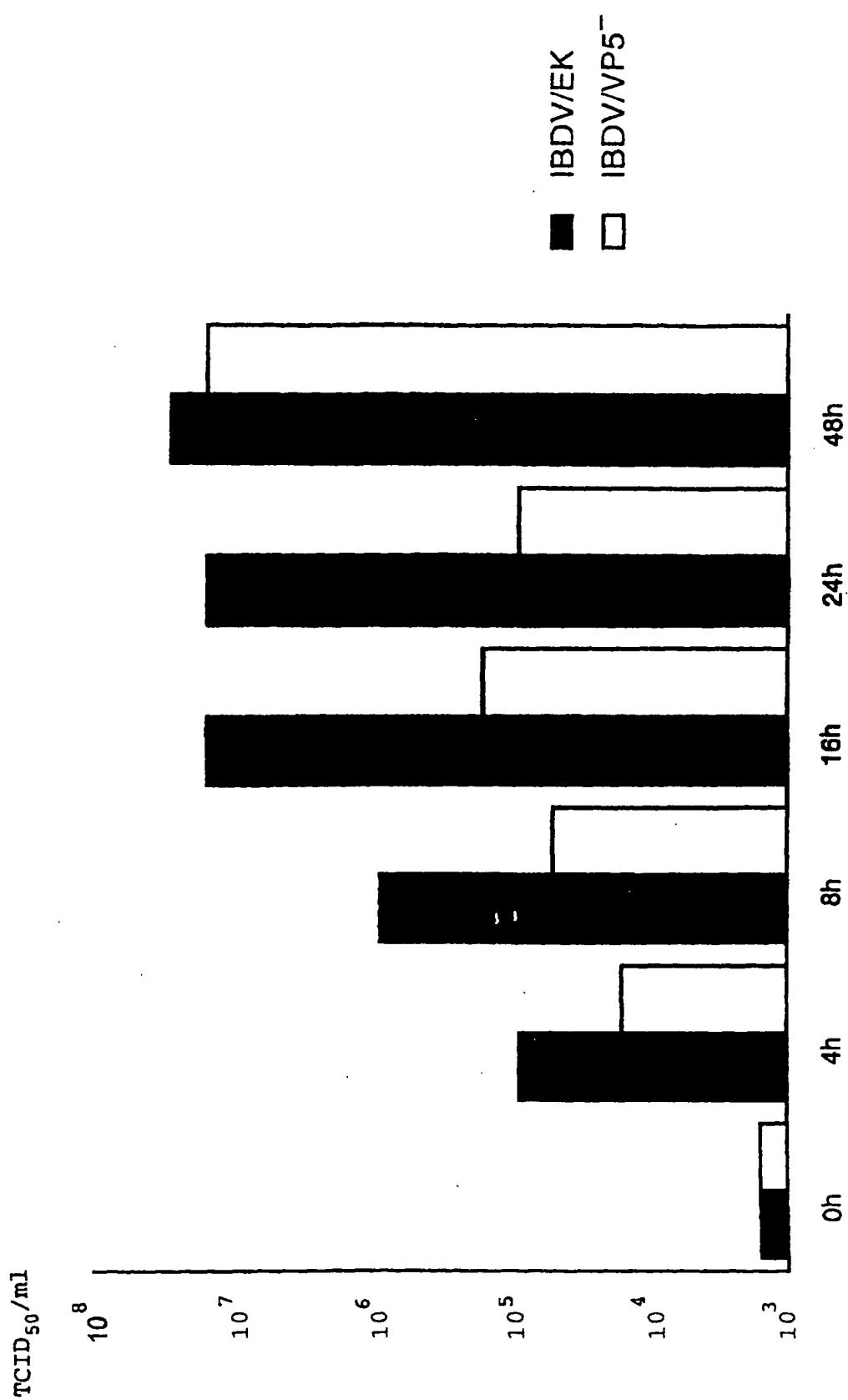


Figure 5



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